Functional Characterization of Anti-idiotypic Antibody Expanded Chimeric Antigen Receptor (CAR) Expressing Re-directed T Cells

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Erklärung:

Ich versichere, dass ich die Dissertation selbstständig gefertigt, alle benutzten Quellen und Hilfsmittel angegeben und Zitate im Text kenntlich gemacht habe. Ich versichere ferner, dass ich die Arbeit weder ganz noch teilweise für eine Prüfung an einer Hochschule eingereicht habe.

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Abstract

Cancer immunotherapy is aimed to activate the immune system to fight cancer. Tumor associated antigens (mostly self-antigens) recognized by cellular or humoral effectors of the immune system are potential targets for an antigen specific cancer therapy. Tumor specific CD8+ cytotoxic T cells play a critical role in the recognition and elimination of tumor cells. Transfusion of *ex vivo* expanded tumor specific T cells in tumor bearing hosts is known as adoptive T cell-therapy. It is widely accepted that cancer cells not only escape from immune recognition but also suppress anti-tumor immune response. Therefore, not all tumors induce tumor specific T cells. To overcome this problem, recombinant T cell-receptors can be expressed in peripheral blood T cells conferring a new specificity. T cells expressing chimeric antigen receptors composed of either antibody-derived Fab or single-chain variable fragment (scFv) as their extracellular recognition elements joined to lymphocyte triggering molecules (e.g. TCR-zeta chain, CD28) are known as re-directed T cells.

The Cancer-Testis (CT) antigen NY-ESO-1 is expressed in a variety of cancers but not in normal adult tissue except for the reproductive tract. NY-ESO-1 is immunogenic, spontaneous humoral and CD8+ T cell responses can be detected in patients with advanced NY-ESO-1 expressing tumors. Due to its tumor-specific expression, it has been targeted by various immuno-therapeutical strategies. Effective cancer immunotherapy depends on the presence of a sufficiently large number of CD8+ lymphocytes with anti-tumor specificity and appropriate homing and effector functions, enabling recognition and destruction of cancer cells. Transfer of *ex vivo* generated CD8+ T cells with chimeric receptors specific for cancer antigens is a promising approach.

In the present study, we expressed chimeric antigen receptors on human CD8+ T cells via retroviral mediated gene transfer. These receptors are composed of single chain variable fragments of various affinities derived from an antibody recognizing the NY-ESO-$1_{157\text{-}165}$ peptide presented in the HLA-A2 complex (TCR like antibody) fused to an intracellular signalling domain of CD28 and CD3 zeta. These re-directed CD8+ T cells show differences in cytotoxicity and IFN-gamma production in response to NY-ESO-1 expressing tumor cells with respect to their affinity. Treatment with the DNA demethylating agent 5-aza-2-deoxycytidine increases or induces the expression of NY-ESO-1 in various tumor cell lines thus enabling or enhancing the effective killing by NY-ESO-1 specific re-directed T cells. We also showed that anti-idiotypic Fab fragments specifically bound to anti-NY-ESO-$1_{157–165}$ CAR and activation of anti- NY-ESO-1 CAR re-directed T cells with anti-idiotypic antibodies was comparable to stimulation with cells

presenting large amounts of HLA-A*0201/NY-ESO-$1_{157–165}$ complexes with respect to functionality *in vitro* and *in vivo* . Stimulation with anti-idiotypic antibodies resulted in 300 fold expansion of re-directed T cells.

Based on our results, we propose the use of high affinity CAR re-directed T cells for adoptive T cell therapy and anti-idiotypic antibodies, which can easily be adapted to GMP regulations, to expand and activate re-directed T cells for therapeutic use.

Zusammenfassung

Es ist bekannt, dass das Immunsystem in der Lage ist, Tumorzellen zu erkennen und abzutöten. Tumor-assoziierte Antigene, die durch die zelluläre oder humorale Immunantwort erkannt werden, sind hierbei potentielle Zielstrukturen für eine antigen-spezifische Immunantwort. Tumor-spezifische CD8+ zytotoxische T-Zellen spielen eine wichtige Rolle bei der Erkennung und Beseitigung von Tumorzellen. Während der Tumorentstehung können die Tumorzellen aber zum einen der Immunerkennung entkommen, zum anderen auch die Immunantwort gegen den Tumor aktiv unterdrücken. Daher konnten nicht bei allen Patienten tumor-spezifische T-Zellen nachgewiesen werden. Um dieses Problem zu überwinden, können rekombinante chimäre T-Zell-Rezeptoren in peripheren Blut-T-Zellen exprimiert werden, die dadurch eine neue Spezifität erhalten, die gegen die Tumorzellen gerichtet ist. Zur Antigenerkennung werden chimäre Antigen-Rezeptoren (CAR) benutzt, die aus Antikörper-abgeleiteten Fab-Fragmenten oder single-chain variablen Fragmenten (scFv) bestehen. Diese CARs sind zur T-Zell-Aktivierung mit Signaldomänen (z.B. TCR-ζ-Kette, CD28) fusioniert. Diese CAR tragenden T-Zellen werden als „re-directed“ T-Zellen bezeichnet. Die Transfusion von *ex vivo* expandierten tumor-spezifischen re-directed T-Zellen in Patienten ist als „Adoptive T-Zell-Therapie“ bekannt und bereits in der klinischen Testung.

Das CT-Antigen NY-ESO-1 wird in einer Vielzahl von Krebsarten exprimiert, aber nicht in normalem adulten Gewebe mit Ausnahme des Keimzellgewebes. Bei einem Teil der Patienten mit fortgeschrittenen NY-ESO-1 exprimierenden Tumoren konnte eine spontane humorale und CD8+ T-Zell Reaktion nachgewiesen werden. Aufgrund seiner tumor-spezifischen Expression dient NY-ESO-1 als Ziel für verschiedene immuntherapeutische Strategien. Eine wirksame Immuntherapie hängt von der Anwesenheit einer ausreichend großer Anzahl tumor-spezifischer und funktionaler CD8+ T-Zellen ab. Um dieses Ziel zu erreichen, ist der adoptive Transfer von *ex vivo* generierten und expandierten CAR tragenden CD8+ T-Zellen ein vielversprechender Ansatz.

In der vorliegenden Studie haben wir CARs in humanen CD8+ T-Zellen mittels retroviralen Gentransfer exprimiert. Diese Rezeptoren basieren auf einem scFv, der spezifisch gegen den HLA-A*0201/NY-ESO-1157-165-Komplex (TCR like antibody) gerichtet war. Hierbei wurden scFv mit verschiedener Affinität getestet. Der scFv wurde fusioniert mit den intrazellulären CD28- und CD3ζ Signaldomänen. Diese re-directed CD8+ T-Zellen zeigten Unterschiede in ihrer Zytotoxizität und Interferon-gamma-Produktion in Abhängigkeit von Antigendichte auf den Tumorzellen und der Affinität des CAR. Zur Steigerung der Antigendichte auf den Zielzellen

wurde die DNA-demethylierende Substanz 5-Aza-2-desoxycytidin getestet. Diese verstärkte oder induzierte die Expression von NY-ESO-1 in verschiedenen Tumorzelllinien, wodurch die NY-ESO-1 spezifischen re-directed T-Zellen eine verbesserte Wirkung zeigten.

Darüber hinaus konnte gezeigt werden, dass anti-idiotypische Fab-Fragmente spezifisch am anti-NY-ESO-1 CAR banden. Basierend auf dieser Beobachtung wurde das Aktivierungsverhalten der anti-NY-ESO-1 CAR tragenden re-directed T-Zellen analysiert, nachdem sie mit Zellen oder dem Anti-Idiotypen stimuliert wurden. Hierbei konnte beobachtet werden, dass die Stimulation mit dem anti-idiotypischen Antikörper vergleichbar war mit Zell-basierter Stimulation, die den HLA-A*0201/NY-ESO-$1_{157\text{-}165}$ Komplex in hoher Dichte exprimierten. Die Stimulierung mit anti-idiotypischen Antikörpern führte zu einer 300-fachen Expansion der re-directed T-Zellen und war somit signifikant grösser als bei der Zell-basierten Expansion. Es gab nach den beiden unterschiedlichen Stimulationen keinen Unterschied zwischen der Funktionalität *in vitro* und *in vivo*. Basierend auf unseren Ergebnissen, schlagen wir die Verwendung von CAR re-directed T-Zellen für die adoptive T-Zell-Therapie vor, die durch die Verwendung von anti-idiotypischen Antikörpern expandiert wurden. Da hierbei das Expansionssystem Zell-frei ist, kann dieser Prozess einfacher GMP-konform durchgeführt werden. Die Expansion und Aktivierung von re-directed T-Zellen soll später für therapeutische Zwecke genutzt werden.

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Abbreviations

ACT	Adoptive cell therapy
AI	Anti-Idiotype
Amp	Ampicillin
APC	Antigen presenting cell
APS	Ammonium peroxodisulfate
bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
CA IX	Carbonic anhydrase IX
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CH1/2/3	Constant heavy chain 1/2/3
CL	Constant light chain
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
d H2O	Deionized water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
EDTA	Ethylene diamine tetra acetic acid

ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
Fab	Fragment antigen binding
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FITC	Fluorescein isothiocyanat
FSC	Forward scatter
g	Centrifugation: earth gravity, weight:gram
Gy	Gray
H	Heavy
HEK	Human Embryonic Kidney
h	Hour
H2SO4	Sulfhuric acid
H3PO4	Phosphoric acid
HCL	Hydrochloric acid
hIgE	Human immunoglobulin E
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
ICOS	Inducible co-stimulator
IFN	Interferon
Ig	Immunoglobulin

IgG	Immunoglobulin G
IL	Interleukin
ITAM	Immunoreceptor tyrosin based activation motifs
kb	Killo base pair
KD	Measure for the affinity of a ligand/analyte system
kDa	Kilo Dalton
LB	Luria Bertain
Le^Y	Lewis Y
MgCl2	Magnesium chloride
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
µg	Microgram
µl	Microliter
min	Minute
ml	Milliliter
mM	Mill molar
MM	Multiple myeloma
mRNA	Messenger ribonucleic acid
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
ng	Nano gram
NK	Natural killer

NOG	NOD scid IL2Rγ deficient mice
NSG	NOD scid gamma
p	P-value
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	R-phycoerythrin
pmol	Picomol
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Incubation: room temperature
s	Second
scFv	Single chain fragment variable
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEREX	Serological analysis of recombinant cDNA expression libraries
SSC	Side scatter
T1	Type 1
TAA	Tumor associated antigen
TAE	TRIS-acetate/EDTA
T_{CM}	Central memory T cell
TCR	T-cell receptor

TE	TRIS-EDTA
T_{EFF}	Effector T cells
T_{EM}	Effector memory T cell
TEMED	Tetramethylen diamine
Th	T helper cell
TIL	Tumor infiltrating lymphocyte
T_M	Memory T cell
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cells
TRIS	Tris-(hydroxymethyl)-aminomethane
U	Unit
UV	Ultraviolet
V	Volume
v/v	Volume per volume
VEGFR	Vascular endothelial growth factor
w/v	Weight per volume
ZAP	Z-associated protein of 70 kDa

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1 Introduction

Hundred years ago, it was postulated that the immune system effectively protects the host from various infections and might also destroy tumor cells. Today it has been widely accepted that the immune system is protecting the host from tumor formation. However, the immunological processes leading to protection are a complex interplay of the tumor tissue and the immune system.

1.1 Immunosurveillance

A fundamental principle of immunosurveillance is the recognition of tumor specific antigens expressed on tumor cells that are different from their parental cells. The hypothesis of immunosurveillance came from the basic experiments performed by Burnet and Thomas (Burnet 1957); they proposed that adoptive immunity was mainly involved in suppressing tumor growth (Stutman 1974; Stutman 1975). The immunosurveillance hypothesis was supported by experiments with immunodeficient mice that showed more susceptibility to induced tumors than immunocompetent mice (Dighe, Richards et al. 1994; Kaplan, Shankaran et al. 1998). Experiments in 2001 demonstrated that the immune system not only controlled the tumor growth but also could support tumor progression. In those experiments, tumor formation was rejected in wild type mice when transplanted tumor cells derived from immunodeficient mice, whereas progressive tumor growth was observed when wild type mice derived tumor cells were used. These results strongly supports that the immune system not only protects but also edits the immunogenicity of the tumor cells (Shankaran, Ikeda et al. 2001; Cheever, Allison et al. 2009).

1.2 Immunoediting

Cancer immunoediting is a complex process, composed of three distinct phases termed elimination, equilibrium and escape (Vesely, Kershaw et al. 2011). During the elimination phase both innate and adoptive immune systems work together to detect and eliminate tumors before they become clinically apparent (Girardi, Oppenheim et al. 2001; Dunn, Old et al. 2004; Guerra, Tan et al. 2008). Although activation of the innate immune system controls the tumor growth, effective immunosurveillance depends on expansion of antigen specific CD4+ and CD8+ T cells (Matsui, Ahlers et al. 1999; Guerra, Tan et al. 2008; Vesely, Kershaw et al. 2011). In the case of ineffective elimination of the tumor cells an equilibrium state between cancer cells and immune cells will be reached. During this phase the immune system controls measurable tumor outgrowth, however tumor cells are present. The equilibrium phase is the longest phase in cancer immunoediting (Aguirre-Ghiso 2007). During the escape phase, tumor cells acquire the

ability to escape from immune recognition and grow to viable and clinical relevant tumors (Dunn, Bruce et al. 2002; Khong and Restifo 2002).

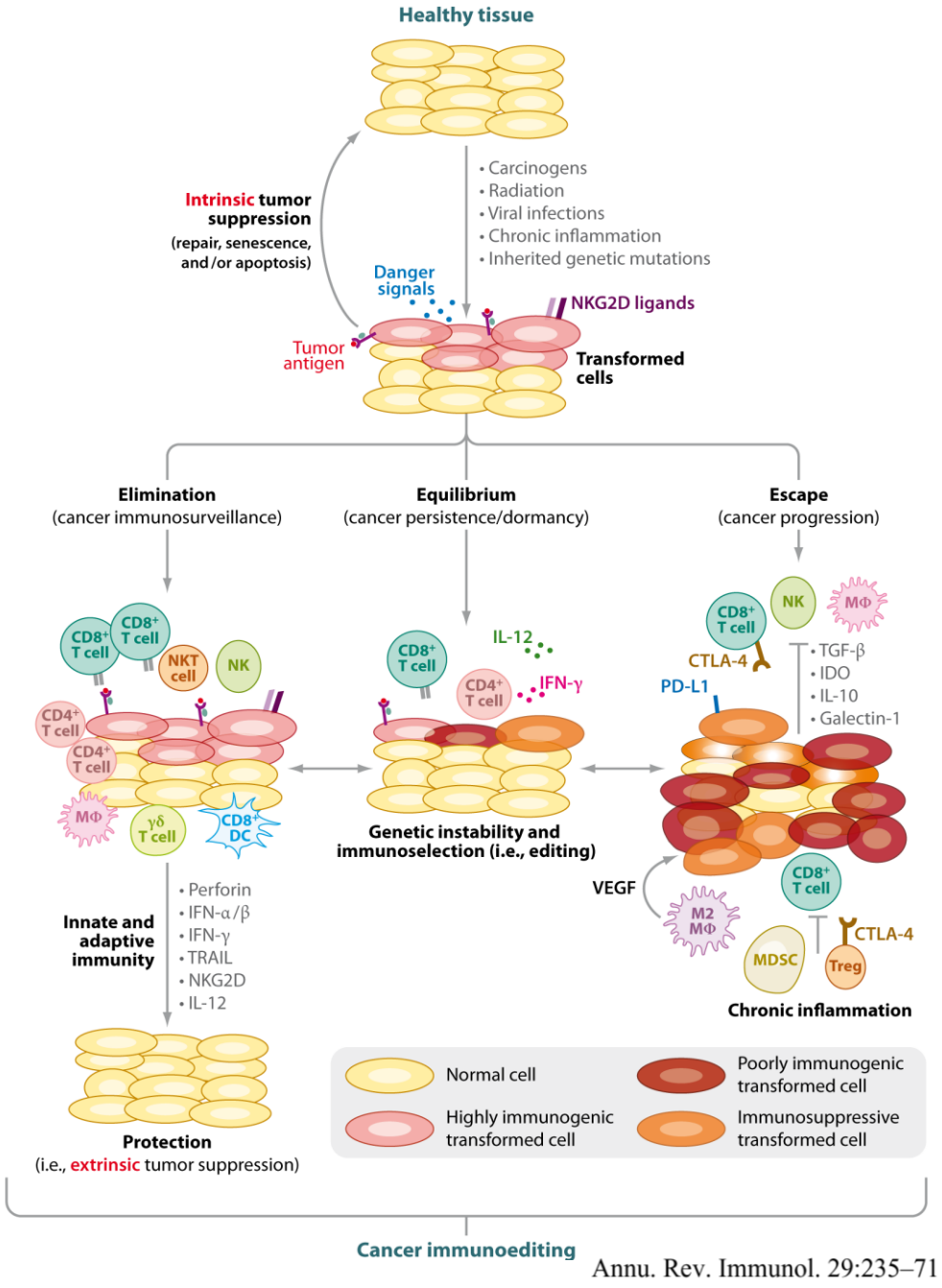

Figure 1-1: Three phases of cancer immunoediting (Vesely, Kershaw et al. 2011)

1.3 Tumor antigens

With the advance of technology, several different tumor associated antigens (TAAs) and tumor specific antigens have been identified (van der Bruggen, Traversari et al. 1991; Traversari, van der Bruggen et al. 1992; Sahin, Tureci et al. 1995). Pfreundschuh and his colleagues have

identified a panel of different antigens with the help of SEREX (Serological analysis of antigen by recombinant expression cloning). SEREX allows the identification of tumor antigens that elicit humoral immune response in cancer patient. Since then, several different tumor antigens have been identified with the help of SEREX (Sahin, Tureci et al. 1995). Based on their expression tumor antigens are classified into different categories as shown in below table (Chen, Scanlan et al. 1997; Scanlan, Gure et al. 2002).

Antigen group	**Antigen name**
Cancer-testis (CT) antigens	MAGE-1,MAGE-2, MAGE-12, BAGE, NY-ESO-1 and CML66
Differentiation antigens	Tyrosinase, TRP-1, TRP-2, gp100 and MART-1
Oncofoetal antigens	CEA, α-fetoprotein and 5T4
Overexpressed antigens	WT-1, MUC-1, proteinase 3 and G250
Mutational antigen	P53
Retroviral antigens	HERV-K10
Splice variant antigens	NY-CO-30

1.4 Cancer-testis antigen NY-ESO-1

NY-ESO-1 is one of the cancer testis (CT) antigens that were first discovered by SEREX (Chen, Scanlan et al. 1997). It was identified in a serum from a patient with squamous cell carcinoma of the esophagus (Chen, Gure et al. 1998). Expression of the NY-ESO-1 protein is strictly restricted to tumor cells as well as immune-privileged regions (germ cells and placenta) (Lethe, Lucas et al. 1998). The molecular weight of the NY-ESO-1 protein is 18KD and contains 180 amino acids. The biological function of the NY-ESO-1 protein is not completely known.

Several studies showed that NY-ESO-1 induces a spontaneous cellular and humoral immune response in patients with metastatic melanoma and other tumor types (Fossa, Berner et al. 2004; Gjerstorff, Kock et al. 2007). Furthermore, it has been demonstrated that NY-ESO-1 elicits both CD8 and CD4 T cell responses in patients with different tumor types, and large number of CD4 and CD8 T cell epitope has been identified (Jager, Chen et al. 1998; Tsuji, Hamada et al. 2008). The NY-ESO-$1_{157-165}$ peptide is one of the immunogenic NY-ESO-1

peptide and induces spontaneous T cell response in patients with different cancers (Yuan, Adamow et al. 2011). In peptide vaccination trails with the NY-ESO-$1_{157-165}$ peptide showed strong CTL response and lysed the tumor cells expressing NY-ESO-$1_{157-165}$ peptide (Tsuji, Hamada et al. 2008).

1.5 T cell responses

Specificity of each T lymphocyte is determined by the expression of a unique T-cell receptor (TCR) on the cell surface. Matured naive T cells migrate to secondary lymphoid organs through blood and lymphatic system where they will be activated by dendritic cells (DCs). Antigen presenting cells (mainly DCs) present antigenic peptides to naïve T cells from infectious agents or self-antigens in the context of MHC molecules. Interaction between an antigen-specific TCR of naïve T cells and a peptide/MHC molecule triggers series of intracellular signaling events resulting in T cell activation and maturation. An antigen specific activated T cell migrates through tissues to the site of infection or antigen presence and mediates cell-mediate effector function (Chtanova, Han et al. 2009). CD8+ T cells are mainly involved in target cell lysis, whereas helper CD4+ T cells produce important cytokines to orchestrate the adaptive and innate immune response. CD8+ T cells recognize peptides that are presented in the context of the MHC class I molecule, whereas helper CD4+ T cells recognize peptide in the context of MHC class II. Antigen specific activated CD8+ T cells release cytokines like IFNγ and TNFα, which enhance the expression of tumor antigens by increasing the expression of MHC class I and class II molecules on tumor cell surface. (Hung, Hayashi et al. 1998; Tosolini, Kirilovsky et al. 2011).(Fefer 1969). CD4 T cells also play critical role in autoimmunity, allergic responses and tumor immunity. During antigen specific activation naïve CD4 T cells may differentiate in to one of the different T helper (Th) cells, including Th1, Th2, Th17 and Tregs in the presence of particular cytokine. Depending on cytokine production, Mosmann and Coffman designated Th1 and Th2 responses (Mosmann, Cherwinski et al. 1986). Th1 cells mainly produce IFNγ and tend to produce IL-2 and TNFα, whereas Th2 fail to produce IFNγ and mainly produce IL-4. In 1995, a third subset of CD4+ T cells was identified with suppressor activity. These cells were termed regulatory T cells or Tregs (Sakaguchi, Sakaguchi et al. 1995). In 2003, fourth effector CD4 T cell population was identified and designated as Th17 cells, known to produce IL-17A,IL-17F and IL-22 cytokines, which are not produced by Th1 and Th2 cells(Park, Li et al. 2005).

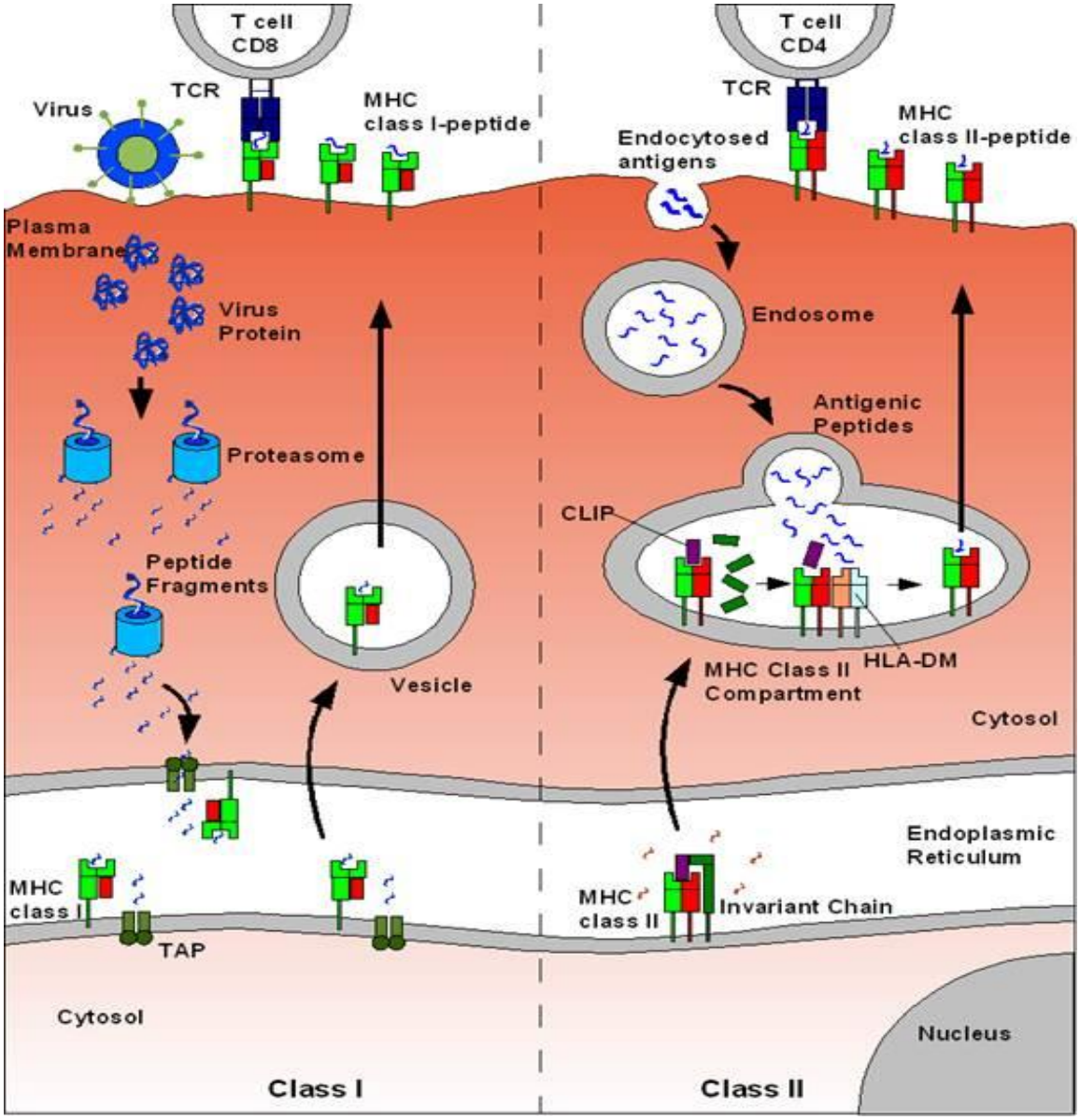

Figure 1-2: Schematic representation of MHC class I (left) and MHC class II (right) antigen presentation pathways *(Janeway CA, Travers P, Walport M, Shlomchik M. Immunobiology. ISBN: 0-4430-7098-9)*.

1.6 Spontaneous T cell response to cancers

Several studies demonstrate that both CD4+ and CD8+ T cell immune responses against tumor associated antigens (TAAs) are spontaneously induced in the phase of cancer immunosurveillance in humans (Vesely, Kershaw et al. 2011). Strong evidence suggests that spontaneous elimination of melanoma lesions mainly depends on the expansion of antigen specific T cells. Several clinical studies showed that antigen specific spontaneous CD4+ and CD8+ T cell response target tumor associated antigens including NY-ESO-1 and MART-1/Melan-A in cancer patients, whereas spontaneous T cell response against other TAAs are rare (Weide, Zelba et al. 2012). Spontaneous tumor regression was observed by lymphocyte

infiltration in various tumor types. Many studies demonstrated that presence of tumor-infiltrating lymphocytes (TILs) in melanoma. Patients with high levels of CD8+ T cells infiltrating the tumor tissues showed prolonged survival when compared to patients with low TIL numbers (Gooden, de Bock et al. 2011). The presence of intra-tumoral CD8+ T cells is associated with superior survival also in squamous cell and adenocarcinomas (Schumacher, Haensch et al. 2001).

1.7 Tumor infiltrating T cells

During the process of the adaptive immune response, T cells migrate and accumulate at the tumor site upon antigen encounter and are called TILs. T cells migrate through tissues and recognize infected cells or cells expressing altered protein molecules in the form of peptides presented on the cell surface through the major histocompatibility complex (MHC)(Deguine, Breart et al. 2010). The main effector function of T cells during the adaptive immune response includes target cell lysis and production of cytokines. Requirements for optimal activation and proliferation of T cells are the recognition of the cognate peptide through the TCR engagement and signaling via co-stimulatory receptors binding to their ligands expressing on antigen presenting cells or tumor cell surface. However, chronic antigen contact leads to exhaustion due to the chronic activation or activity T cells (Moskophidis, Lechner et al. 1993).

Furthermore, TILs encounter immunosuppressive mechanisms at the tumor site (Restifo, Marincola et al. 1996; Schreiber, Old et al. 2011). Advanced technologies are available allowing the culturing and manipulating of T cells for improving their therapeutic efficacy. TILs can be isolated from tumor tissues and *in vitro* culturing could restore their activity. Adoptive transfer of fully functional *in vitro* expanded TILs has shown promising clinical response (Dudley, Wunderlich et al. 2002). However, generation and expansion of antigen specific TILs to clinically relevant numbers is technically challenging due to very limited number of tumor antigen specific T cells. Another limitation in regards to TILs is the fact that not in all tumors tissues TILs can be found or transferred in *ex vitro* culture systems.

1.8 Re-directed T cells

An alternative to the TIL strategy is the use of gene therapy by cloning TCRs or chimeric antigen receptors (CAR) into vectors and express them in autologous T cells. These re-directed T cells recognize the antigen with the help of the new receptor resulting in the redirection of the T cells.

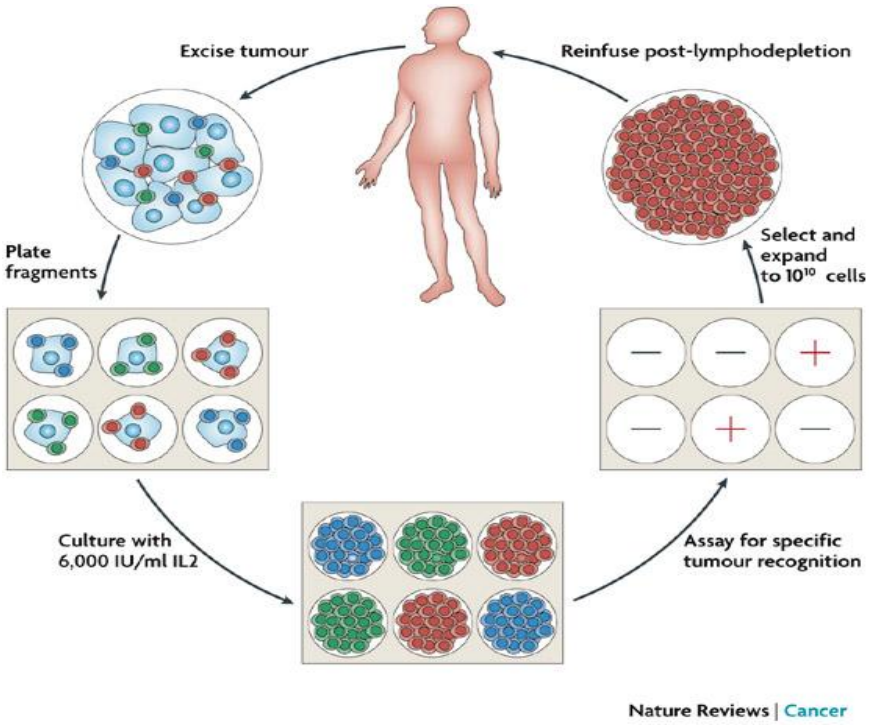

Figure 1-3: Generation of anti-tumour T cells

A tumor is excised and multiple individual cultures are established, separately grown and assayed for tumor specific T cells. Cultures with high anti-tumor reactivity are expanded to large number (>10^{10} cells) and re-infused into the patient (Rosenberg, Restifo et al. 2008) .

1.8.1 Chimeric Antigen Receptor (CAR)

The main drawback of adoptive T cell therapy is that tumors frequently lose antigen expression by down regulating MHC expression (Yadav, Ngolab et al. 2009), which makes the use of recombinant re-directed T cells an attractive approach. T cells can be re-directed using genes that encode monoclonal antibody chains or TCR like antibodies selected from phage display library (Held, Matsuo et al. 2004). These recombinant immunoreceptors are also known as chimeric antigen receptor (CAR). The idea of designing an chimeric antigen receptors came from the basic experiments performed in the early 1990`s, demonstrating that recombinant receptors made by fusing antibody domains with TCRβ, could direct the T cell activation independent of TCR-MHC interaction (Eshhar, Waks et al. 1993). The generation of CARs has been developed as a tool to re-direct T cells to target surface expressing tumor antigens to overcome immune surveillance mechanism by which tumors escape from endogenous T cells by losing MHC molecules. CARs are composed of an antigen recognizing region (mostly scFv) mainly derived from a monoclonal antibody fused with intracellular T cell signaling domains and transmembrane domain for optimal cell surface expression (Hombach, Wieczarkowiecz et al. 2001; Heuser, Hombach et al. 2003).

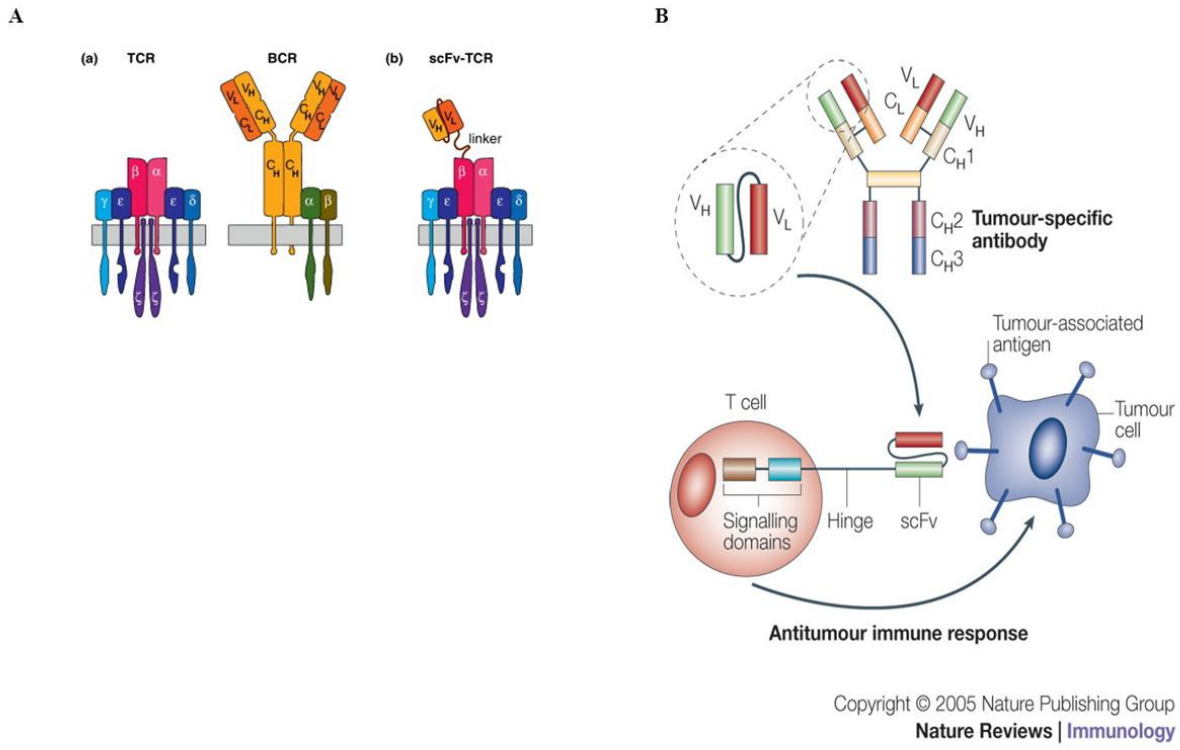

Figure 1-4: **A** Natural and synthetic antigen receptors **(**Schamel and Reth 2012)**. B** Schematic representation of a typical chimeric antigen receptor(Kershaw, Teng et al. 2005).

1.8.2 T-cell Receptor (TCR)

Antigen expressing target cells are recognized by a T cell with the help of special T cell receptors (TCR) expressed on the cell surface. TCRs are heterodimers composed of two highly variable α and βchains, paired with each other and the amino acid sequences of both α and β chains are responsible for antigen specificity (Viola and Lanzavecchia 1996). Tumor specific re-directed T cells can be established by identifying and isolating TCRs from the T cells clones generated for defined target antigen (Abad, Wrzensinski et al. 2008). Recent molecular biology techniques allow for cloning of the genes that encode α and β chains of the antigen specific TCRs by using viral vectors (Sadelain, Riviere et al. 2003). In order to generate functionally competent re-directed T cells, both TCRα and β chain should be expressed efficiently. Optimal expression and pairing of both TCRα and β chains can be achieved by linking both chains to 2A ribosomal skip peptide. Cloning of antigen specific TCRs allows for re-directed T cells to respond antigen expressing tumor cells. The main disadvantage with α and β chain transfer is the miss-pairing with endogenous TCR chains (Kuball, Dossett et al. 2007). This miss-pairing may lead to reduced T cell activation and unintended autoimmune reactivity. Other draw-backs with the αβ TCR based re-directed T cells therapy is restriction to single HLA epitope.

Furthermore, TCRs cannot target whole proteins expressed on cell the surface and non-protein tumor antigens (Hiasa, Hirayama et al. 2008).

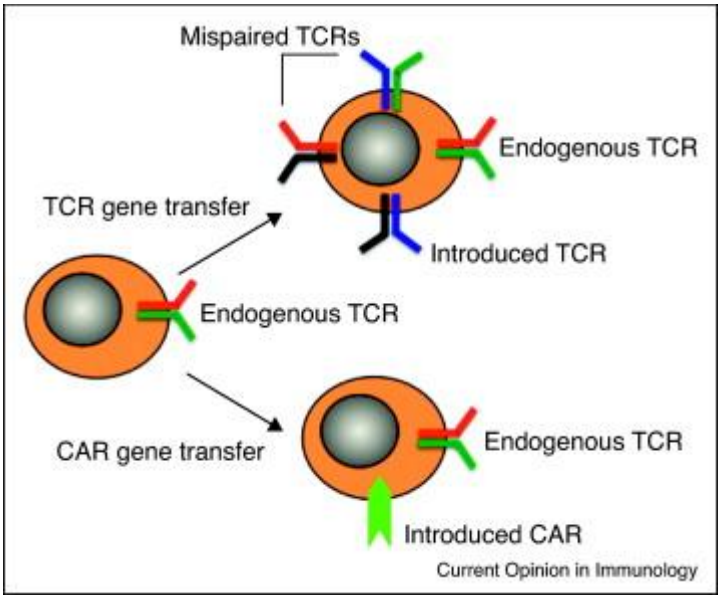

Figure 1-5: Re-directing T cells specificity by insertion of genes encoding a TCR or CAR (Turtle and Riddell 2011).

1.8.3 Ligand

In addition, ligands have also been shown to be useful for the use of TCRs and monoclonal antibodies to re-direct T cells (Lehner, Gotz et al. 2012; Morgan, Johnson et al. 2012). This is especially a powerful tool for the destruction of tumor vasculature. Recruiting of new blood vessels is the main requirement for the growth of solid tumors and, thus, inhibition of angiogenesis by using monoclonal antibodies or small molecules that interfere in their formation showed promising data (Muniappan, Banapour et al. 2000). An alternative strategy is to completely destroy the newly formed blood vessels by targeting endothelial growth factor receptors with their ligands or monoclonal antibodies conjugated with toxins. Using ligands as a target-binding moiety for constructing CARs, complete destruction of tumor vasculature could be achieved (Niederman, Ghogawala et al. 2002; Kahlon, Brown et al. 2004).

1.8.4 Signalling via chimeric antigen-receptor

CAR construction requires four key components: An antigen specific moiety, an extracellular spacer molecule, a transmembrane domain and, finally, intracellular T cell signaling molecules. In general, an antigen specific moiety is a scFv derived from a tumor specific or tumor associated antigen specific monoclonal antibody. In some cases, ligands also are used for cloning CARs to target tumor vasculature. The architecture of a scFv is composed of a variable light (VL) and variable heavy (VH) chain of the monoclonal antibody joined by a flexible linker. A hinge or extracellular spacer molecule provides flexibility and accessibility for the scFv or binding molecule. Most commonly used extracellular spacer molecules are immunoglobulin fragment crystallizable regions (Fc) or extracellular domains of the CD8α or CD28 molecule, respectively. Transmembrane domains are usually derived from the most proximal molecule of the intracellular signaling domain. Primarily signaling domains derived from the CD3ζ (ITAM) chain or FcεRIγ are used. However, CD3ζ intracellular signaling domains have been shown to provide superior *in vivo* anti-tumor efficacy than FcεRIγ (Hombach, Wieczarkowiecz et al. 2001; Heuser, Hombach et al. 2003). CARs containing a single CD3ζ domain as intracellular signaling molecule are called first generation CARs. Although, first generation CARs can mediate cytotoxicity *in vitro*, they fail to confer T-cell sustainability for longer periods *in vivo*. It is known that for fully activated T cells, a co-stimulatory signal is required via CD28-B7 interaction in addition to CD3ζ signaling. Incorporation of a CD28 co-stimulatory intracellular domain in CARs resulted in sustained proliferation of re-directed T cells *in vitro* and *in vivo*. These CARs are called second generation CARs (Harding, McArthur et al. 1992; Chambers and Allison 1997; Finney, Akbar et al. 2004). Third generation CARs were developed with three different intracellular signaling domain molecules. Most commonly used third intracellular signaling molecules are OX40 and CD137 (4-1BB). Third generation CARs have been shown to increase proliferation of re-directed T cells and secretion of IL-2 *in vitro* (Till, Jensen et al. 2012).

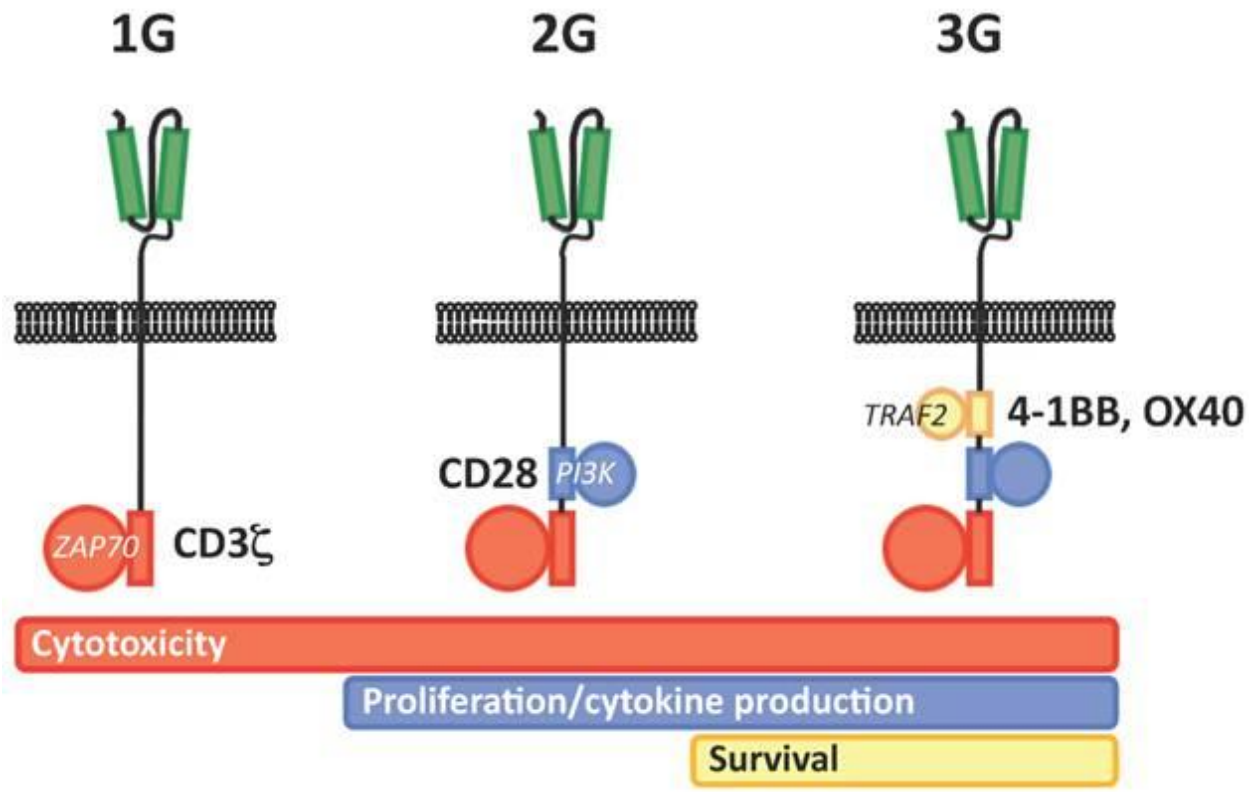

Figure 1-6: Schematic representation of the different generation s of CARs

1G, first generation, 2G, second generation, 3G, third generation CAR. The scFv is highlighted in green, while the different components of TCR signal transduction machinery are highlighted in red (CD3ζ chain/ZAP 70), blue (CD28/PI3K) and yellow (4-1BB or OX40/TRAF) (Casucci and Bondanza 2011).

1.8.5 Clinical use of re-directed T cells

The critical evaluation of re-directed T cells *in vitro* and several preclinical models have shown promising results encouraging the use of re-directed T cells in adoptive T cell transfer in clinics. First successful anti-tumour responses with re-directed T cells were observed in patients treated with anti-MART TCR re-directed T cells (Morgan, Dudley et al. 2006). In this study, two of the fifteen patients demonstrated regression of metastatic melanoma lesions after one year of infusion of the circulating re-directed T cells. This was the first clinical evidence that showed that gene modified re-directed T cells can mediate anti-tumor effects. A recent clinical trial with NY-ESO-1 specific TCR transduced re-directed T cells have showed promising results in melanoma and synovial cell sarcoma patients expressing the NY-ESO-1 antigen (Robbins, Morgan et al. 2011). In this study, two of 11 patients showed complete regression. Phase I clinical trials with first generation CARs have shown very little anti-tumor activity and poor persistence after infusion of re-directed T cells. First promising clinical responses were observed with second

generation CARs targeting CD19 antigen in a patient with chronic lymphocyte leukemia (van der Bruggen, Traversari et al. 1991; Shankaran, Ikeda et al. 2001; Yadav, Ngolab et al. 2009; Porter, Levine et al. 2011; Robbins, Morgan et al. 2011; Schreiber, Old et al. 2011; Till, Jensen et al. 2012). Currently, several clinical studies are being evaluated with second and third generation CAR re-directed T cells (www.clinicaltrial.gov; NCT00840853, NCT00673829)

1.8.6 Side effects

No clinical evidence has been documented for insertional mutagenesis with viral mediated gene transfer in re-directed T cells (Nicholson, Ghorashian et al. 2012) in retroviral insertional mutagenesis in stem cell therapy for X-linked SCID (Hacein-Bey-Abina, Von Kalle et al. 2003). The main safety issues with TCR re-directed T cells are pairing of newly introduced TCRs with endogenous TCRs, which may result in unknown specificity. However, studies with TCR re-directed T cells mainly focused on preventing miss-pairing by engineering TCRs. Major concern with CAR re-directed T cells is the selection of the target antigens, since the majority of the tumor associated antigens (TAAs) though are over-expressed on tumor cells, however, have been observed also to have basal expressed on normal tissues. More recently, a clinical study with carbonic anhydrase IX (CA IX) specific CARs induced adverse effects (Lamers, Sleijfer et al. 2006; Lamers, Willemsen et al. 2011). In this study, patients with metastatic renal cell carcinoma (RCC) were treated with anti-CA IX CAR re-directed T cells. Some of them have developed severe liver toxicity because of T cells targeting the CAIX antigen also present in bile ducts (Lamers, Sleijfer et al. 2006). Second and third generation CAR re-directed T cells have induced signals which are intended to prolong survival and enhance proliferation upon antigen encounter. Due to the basal expression of antigen in normal tissue like described for HER2/neu, the encounter of antigen with CAR re-directed T cells can cause on-target off side adverse effects (Morgan, Yang et al. 2010). All these data taken together suggest that selection of the target antigen is the most crucial step in the development of CAR mediated adoptive T cell therapy.

1.8.7 Therapeutic efficacy

There is a rapidly growing number of clinical trials testing the concept of adoptive T cell therapy as a treatment option in cancer patients who have not responded to conventional therapies. Genetically engineered TCRs that prevent miss-pairing with endogenous TCRs, selection of different T cell subtypes and generation of second and third generation CARs have improved the scope of adoptive T cell therapy with re-directed T cells (Kohn, Dotti et al. 2011). Through technical improvement leading to reduced miss-pairing with endogenous TCRs and improved affinity for the antigen are additional advantages for the TCR re-directed T-cell therapy (Ferrara,

Reddy et al. 2010). Lymphocyte depletion prior to infusion of re-directed T cells have shown to improve anti-tumour effect in both preclinical and clinical settings (Restifo, Dudley et al. 2012)

1.9 Expansion of T cells

Several different approaches have been developed to generate tumor specific T cells. However, effective clinical response of TILs or re-directed T cells always depends on the number and quality of T cells generated *in vitro*. Major limiting factor is the large-scale number required to overcome inhibitory mechanism in the tumor microenvironment. Therefore, strategies allowing large-scale expansion of functionally intact re-directed T cells under GMP conditions have to be developed. T-cell activation and proliferation are mainly mediated by the CD3/TCR complex and co-stimulatory molecules such as CD28 and the signals are mainly provided by antigen presenting cells (APCs) (Durai, Krueger et al. 2009). Recently, several methods have been proposed to increase the number of tumor-specific T cells, which rely on the stimulation with antigen presenting cells pulsed with specific peptide, artificial antigen presenting cells or the polyclonal stimulation using either lectins or anti-CD3 plus anti-CD28 antibodies, respectively (Li and Kurlander 2010; Brimnes, Gang et al. 2012). Polyclonal stimulation with anti-CD3 plus anti-CD28 results in the expansion of all cells (tumor-specific and polyclonal T cells) leading to large cell numbers with unknown specificity bearing the risk of off-target toxicity. The expansion of T cells with antigen presenting cells is technically challenging, more difficult to standardize and, therefore, not a straightforward GMP approach.

2 Aim of the thesis

The aim of this thesis is to analyse T cells expressing a chimeric antigen receptor (CAR) derived from a TCR like antibody targeting NY-ESO-1 in regards to functional consequences of the interaction between the CAR and the antigen. Immunotherapy using re-directed T cells has emerged as a powerful tool for the treatment of cancer. The Cancer-Testis (CT) antigen NY-ESO-1 is expressed in a variety of cancers but limited in expression on normal cells. NY-ESO-1 protein has been used as a target for different cancer immunotherapies like peptide vaccination and adoptive transfer of antigen specific re-directed T cells. *In vitro* generation of chimeric antigen receptor (CAR) re-directed T cells with defined antigen specificity is an established method for cancer immunotherapy. However, selection of target antigens and characterization of CAR re-directed T cells is crucial for successful clinical outcomes. For better clinical efficacy, CARs have to be expressed efficiently on re-directed T cells and should have sufficient affinity to their target antigen but recognize only few molecules of endogenously processed peptides on the tumor cell surface without cross-reactivity. Previously, our group has been successful in selecting Fab molecule specific for the HLA-A2/NY-ESO-$1_{157-165}$ peptide using a phage display library. The objective of this study was to determine the relevance of CAR affinities in generating re-directed T cells specific for NY-ESO-1 positive tumors and the feasibility of using anti-idiotypic Fab molecules for the selective expansion of anti-NY-ESO-1 CAR re-directed T cells in sufficient numbers for adoptive transfer. In order to determine the interaction between the peptide specific anti- NY-ESO-1 CAR and the NY-ESO-1 antigen and the resulting cellular processes following goals were specified.

- Expression of anti-NY-ESO-1 CAR constructs with different affinities that recognize HLA-A2/NY-ESO-$1_{157-165}$ peptide on T cells
- Characterization of re-directed T cells *in vitro*
- Characterisation of CAR re-directed T cell activation in respect to antigen density
- Anti-idiotypic Fab antibody dependent expansion of CAR re-directed T cells
- Phenotypical and functional analysis of anti-idiotypic Fab antibody expanded CAR re-directed T cells *in vitro*
- Anti-tumor efficacy of *in vitro* expanded re-directed T cells *in vivo*

3 Material and Methods

3.1 Equipment

Agarose Gel Documentation	BioDoc-It Imaging System	UVP, Upland, USA
Autoclave	V-100	Systec, Hünenberg , Switzerland
Blotting apparatus	Trans-Blot SD semi-Dry Transfer cell	Bio-Rad, Hercules USA
Cell counter	CASY Cell Counter Model TT	Roche Innovatis, Bielefeld, Germany
Centrifuges	Centrifuge 5804R Centrifuge 5810R Centrifuge 5415D	Eppendorf, Hamburg, Germany
Flow cytometers	FACScan FACSCalibur FACSCanto II	BD Biosciences, San Jose, USA
Magnetic-activated cell sorting	MACS multistandard &quadro MACS	Miltenyi Biotec, Bergisch Gladbach, Germany
PCR cycler	T300 Thermocycler	Biometra, Göttingen, Germany
Plate reader	Wallac Victor2 1420 Multilabel Counter	Perkin Elmer, Waltham, USA
Shaker system	Multitron 2	Infors HAT, Bottmingen, Switzerland

Microscope	Polyvar 2	Leica reichert jung, Heerbrugg, Switzerland
Spectrometer	BioPhotometer	Eppendorf, Hamburg, Germany
Water Purification	Milli-Q Gradient System	Millipore, Bedford, USA
Western blot detection unit	ChemiDoc-It Imaging System With BioChemi HR Camera	UVP, Upland, USA

3.2 Kits

QIAGEN Plasmid Maxi Kit	QIAGEN, Hilden, Germany
QIAprep Spin Miniprep Kit	QIAGEN, Hilden, Germany
QIAquick Gel Extraction Kit	QIAGEN, Hilden, Germany
MycoAlert Mycoplasma Detection Kit	Lonza Cologne GmbH, Cologne, Germany
BD Cytofix/Cytoperm Kit	BD Biosciences, San Diego, USA
BD OptEIA Set Human IFNγ	BD Biosciences, San Diego, USA
BD OptEIA Set Human IL-2	BD Biosciences, San Diego, USA

3.3 Buffers

TAE	2 mM 0.25 mM 0.5 mM	TRIS Ultra Acetic acid EDTA
PBS	150 mM 10 mM 1.5 mM	NaCl Na_2HPO_4 KH_2PO4
0.05 % PBST	0.05 % (v/v)	Tween20 99.95 % (v/v)PBS
Flow cytometry buffer	2 % (v/v) 0.01 % (w/v) 5 mM 1 l	FBS, heat inactivated Sodium azide EDTA PBS
Blocking solution	10 % (v/v) 90 % (v/v)	FBS, heat inactivated PBS
TE	10 mM 1 mM	TRIS Ultra EDTA Adjust to pH 8
5X SDS loading buffer	0.28 M 1 % (w/v) 30 % (v/v) 0.0012%(w/v) 5.5 % (v/v)	TRIS Ultra SDS Glycerol Bromophenol blue B-mercaptoethanol

1.5 M TRIS/SDS pH 8.8	1.5 M 0.4 % (w/v)	TRIS Ultra SDS Adjust to pH 8.8
0.5 M TRIS/SDS pH 6.8	0.5 M 0.4 % (w/v)	TRIS Ultra SDS Adjust to pH 6.8
12% (v/v) running gel	35 % (v/v) 40 % (v/v) 25 % (v/v) 0.01 % (v/v) 0.0005 % (v/v)	dH20 Acrylamide (30 %) 1.5 M TRIS/SDS pH 8.8 APS TEMED
5% (v/v) stacking gel	61 % (v/v) 13 % (v/v) 25 % (v/v) 0.01 % (v/v) 0.0007 % (v/v)	dH20 Acrylamide (30 %) 0.5 M TRIS/SDS pH 6.8 APS TEMED
SDS running buffer	25 mM 0.1% (w/v) 200 mM	TRIS Ultra SDS Glycine
Coomassie destain	45 % (v/v) 10 % (v/v) 45 % (v/v)	Methanol Acetic acid (99%) dH2O

Western blot transfer buffer	25 mM 190 Mm 20 %	TRIS Ultra Glycine Methanol
5X DNA loading dye	50% (v/v) 50 % (v/v) 0.4 % (v/v)	TAE Glycerol Orange G

3.4 Antibodies

Human OKT3	eBioscience, San Diego, USA
Human CD28 (co-stimulatory)	eBioscience, San Diego, USA
Anti-human CD8-FITC	eBioscience, San Diego, USA
Anti-human IgG-PE	Southern Biotech, Birmingham, USA
Biotin-AffiniPure F(ab')2 fragment goat anti-human IgG (H+L)	Jackson Immuno Research, Suffolk, UK
Anti-human CD8 microbeads	Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-human HLA-A, B, C-FITC	Biolegend, San Diego, USA
Mouse IgG2a, κ – FITC Isotype control	Biolegend, San Diego, USA
Anti-human IgE antibody	Biolegend, San Diego, USA
Anti-human CD62L-FITC	BD Biosciences, San Jose, USA

Anti-human CD45RA-PB	Biolegend, San Diego, USA
Anti-human IFNγ-FITC	BD Biosciences, San Jose, USA
Anti-human TNFα-APC	Biolegend, San Diego, USA
Anti-human IL-2-PB	Biolegend, San Diego, USA
Mouse monoclonal penta –His antibody	QIAGEN, Hilden, Germany
Mouse Anti-NY-ESO-1 clone E978	Invitrogen Corporation, Camarillo, USA

3.5 Tetramer

PE-conjugated HLA-A2/NY-ESO-1 $_{157-165}$ tetramer	LICR, Lausanne, Switzerland

3.6 Fab molecules

Anti-idiotype Fab A4 and H6	In house, Zurich, Switzerland
Control Fab (B1)	In house, Zurich, Switzerland

3.7 Cytokines

Recombinant human IL-2	Immuno Tools, Friesoythe, Germany
Recombinant human IL-15	Immuno Tools, Friesoythe, Germany

3.8 Peptides

Primer designation	Primer designation
NY-ESO-1 $_{157-165}$ peptide	SLLMWITQV
Influenza matrix protein $_{58-66}$	GILGFVFTL

3.9 Enzymes

Restriction enzyme NcoI	New England BioLabs, Ipswich, USA
Restriction enzyme NcoI	New England BioLabs, Ipswich, USA
Streptavidin-Horse Radish Peroxidase	BD Biosciences, San Diego, USA

3.10 Plasmids and bacteria

3M4E5-CD28/CD3ζ (1046wt) or anti-NY-ESO-1 CAR	Expression cassette codes for a fusion protein consisting of a Lκ-leader sequence, the wt 3M4E5 scFv recognizing the HLA-A*02:01/NY-ESO-$1_{157-165}$ complex, a human CH_2CH_3 IgG domain and a CD28/CD3ζdomain.	Stewart-Jones et al., 2009
T1-CD28/CD3ζ(# 1190) or anti-NY-ESO-1 CAR-T1	Expression cassette codes for a fusion protein consisting of a Lκ-leader sequence, T1 scFv recognizing the HLA-A*02:01/NY-ESO-$1_{157-165}$ complex, a human CH_2CH_3 IgG domain and a CD28/CD3ζdomain.	Stewart-Jones et al., 2009

BW431/26-CD28/CD3ζ (#607) or anti-CEA CAR	Plasmid coding for a CEA recognizing chimeric antigen receptor expressing CD28 and CD3 Expression cassette codes for a fusion protein consisting of a Lκ-leader sequence, a scFv recognizing carcinoembryonic antigen (CEA), a human CH_2CH_3 IgG domain and a CD28/CD3ζ domain.	Kind gift from Prof. Abken, Cologne, Germany
pCOLT-GalV (#392) Helper plasmid	Retroviral helper plasmid coding for GALV env protein	Kind gift from Prof. Abken, Cologne, Germany
pHIT60 (#393) Helper plasmid	Retroviral helper plasmid coding for MLV gag and MLV pol protein	Kind gift from Prof. Abken, Cologne, Germany
pCES1 plasmid	pCES1 containing the variable domains of HC and LC fragments of selected anti-idiotypic Fab antibodies A4 and H6	Kindly provided by Dyax, MA, USA

3.11 Media for bacteria

LB medium	10 g 5 g 5 g 1 L	Tryptone Yeast extract NaCl dH2O
LB-AMP	LB medium with 100 µg/ml ampicillin	Sigma, St.Louis, USA
LB-Amp Plates	LB medium with 1.5 % (w/v) Difco agar	BD, Sparks, USA
2xTY	16 % (w/v) 10 % (w/v)	Bacto™ Tryptone yeast extract

	5 % (w/v)	NaCl
2xTY-AG	0.1 mg/ml 2 % (w/v)	2xTY ampicillin glucose
2xTY-AG agar plates	1.5 % (w/v)	2xTY-AG Difco™ Agar

3.12 Bacterial strain

One shot TOP10 Chemically Competent *E.coli*	Invitrogen, Karlsbad, USA
Premade Z-competent *E.coli* cells (TG-1)	Zymo Research, Irvine, USA

3.13 Primers

Primer designation	**Primer sequence**
pCES fwd_LC	5' – AGC GGA TAA CAA TTT CAC ACA GG - 3'
pCES rev_HC	5' – GGA AGT AGT CCT TGA CCA - 3'
Fwd-NcoI-Part1Lka	CGT ACC ATG GAT TTT CAG TGT CAG ATT TTC AGC TTC TTC ATG CTA ATC AGT GCC TCA GTC ATA ATG TC
Rev-BamH1-3M4E5	GAT AGG ATC CAC TGT GGG GTT GG

3.14 Cell culture media and cell lines

All Cell lines were maintained at 37 °C in a CO2 incubator. Cell culture reagents were purchased from Gibco/Invitrogen (Carlsbad, USA) and Merck (Darmstadt, Germany).

3.14.1 Cell culture media

Fetal Bovine Serum (FBS) Heat inactivated at 56 °C for 30 min	Gibco, Carlsbad, USA
RPMI 1640 media with Glutamax	Gibco, Carlsbad, USA
Hygromycine B	Gibco, Carlsbad, USA
Pencillin/Streptomycine	Gibco, Carlsbad, USA
0.05 % Trypsin-EDTA	Gibco, Carlsbad, USA
Dimethylsulfoxide (DMSO)	Merck, Darmstadt, Germany

3.14.2 Cell line

293T	Derived from 293 cells, carries the SV40 large T antigen	Kind gift from Prof. Abken, Cologne, Germany
T2-1B	Minigene-transfected T2 cell line expressing NY-ESO-$1_{157-165}$/HLA-A2 complex	Held et al., 2004
T2-1C	Minigene-transfected T2 cell line expressing NY-ESO-$1_{155-163}$/HLA-A2 complex	Held et al., 2004
U266	Human multiple myeloma cell line, secrete hIgE molecules	Kind gift from Prof. Dr. U. Keilholz, Charitè, Berlin, Germany
MCF7	Breast cancer cell line	Kind gift from Prof. M.Van den Broek, Zürich, Switzerland

3.15 Mice

Inbred NOD-SCIDγc $^{-/-}$ (NSG) mice were maintained under specified pathogen-free conditions in our Institutional Animal Care unit. 6-8 weeks old NSG mice were used *for in vivo* experiments

3.16 Software

GraphPad Prism V 5.01 for windows	GraphPad Software, San Diego, USA
Cellquest Pro 4.0.2	BD Biosciences, San Jose, USA
FACSdiva	BD Biosciences, San Jose, USA
FlowJo 7.2.5	Tree Star, Ashland, USA

3.17 Bacterial methods

3.17.1 Transformation of competent *E. coli* cells

The ligation mixture was added to 1 vial of commercially available TOP10 competent *E. coli* cells and incubated for 15 min on ice, followed by heat-shock for 45s at 42 °C and immediately placed on ice for 1 min. 250 µl SOC medium was added followed by shaking for 1 hour at 37 °C at 250 rpm.

3.17.2 Growth of bacteria on agar plates

50 µl of freshly transformed *E.coli* cells or glycerol stock diluted in LB-Amp medium was plated on LB-Amp agar plates. Plates were incubated over night at 37 °C.

3.17.3 Growth of bacteria in suspension

For small scale bacterial expansion, single colony from LB-Amp agar plate or 15 µl of bacterial glycerol stock was inoculated in 5 ml of LB-Amp media and incubated over night at 37 °C in a shaker (200 rpm)

3.17.4 Glycerol stocks of bacteria

15 % v/v of glycerol was mixed with overnight grown bacterial culture in 2 ml cryotube and stored at -80 °C.

3.18 Molecular biology techniques

3.18.1 Purification of plasmid DNA from *E.coli*

Large amounts of plasmid DNA required for transient transfections was purified from overnight grown TOP10 strain carrying plasmid of interest. QIAGEN plasmid Maxi Kit was used to extract DNA was extracted from QIAGEN plasmid Maxi Kit according to manufactures protocol. DNA was eluted with sterile molecular biology grade water. DNA concentration was determined by using Nano Drop.

3.18.2 Agarose gel electrophoresis

To check the purity of the plasmid DNA, 0.7-1 % (w/v) agarose (promega, Madison, USA) gel was prepared with TAE buffer, 10 µg/ml of ethidium bromide was added to the gel. Purified DNA samples were mixed with DNA loading dye and 100 bp or 1 kb DNA ladder was used as size standard. 180-100 V of current was applied to the gel and the separation was monitored under UV light.

3.18.3 Expression and purification of anti-idiotypic Fab antibodies

Overnight grown *E.coli* TG-1 bacterial cultures expressing anti-idiotypic Fab antibodies were inoculated at 1:100 dilutions in 1 l of fresh 2xTY broth, containing 100 µg/ml ampicillin and 0.1 % glucose and grown at 37 °C. Cells were induced with IPTG when OD 0.8-1 was reached and continued to grow at 30 °C for 4 h. Cells were then centrifuged at 4000 rpm for 15 min. and peri-plasmic proteins isolated by osmotic shock. His-tag containing Fabs were purified by using Talon Metal affinity resin (Clontech, Mountain View, CA). Purity of Fab was accessed by 12 % SDS gel electrophoresis.

3.18.4 SDS Polyacrylamide gel electrophoresis

2 µg of purified Fab antibody molecules in 20 µl were mixed with 5 µl of 5X loading buffer and denatured at 95 °C for 5 min. proteins were first collected in a 5 % (v/v) stacking gel at 45 mA and subsequently separated according to their molecular weight in 12 % running gel at 70 mA using running buffer as previously described in buffer section. Precision Plus ProteinTM dual color protein marker was used as molecular weight standard. Gels were stained with Coomassie staining solution and gels were de-stained with Coomassie de-stain until protein bands were visible.

3.18.5 Western blotting

For the detection of proteins, unstained gels and PROTRAN nitrocellulose membrane (0.45 µm pore size) were equilibrated in transfer buffer and proteins from separated gel were blotted using a Trans-blot SD apparatus for 30 min at 12 V per gel. For the detection of designated target proteins, membrane was blocked with 4 % MPBS for 1h at room temperature. Membrane was incubated with specific antibodies in PBS for 1h; membrane was washed with 0.1 % PBS-T for 10 min and incubated with anti-mouse/HRP conjugate for 1 hour. After 10 min washing, detection of protein was performed using Amersham ECL western blotting detection reagents.

3.19 Cell culture and immunological methods

3.19.1 Cultivation of cell lines

293T, U266 and MCF7 cells were cultivated in R10 media (RPMI 1640 GlutaMax supplemented with 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin). NY-ESO-1 peptide transfected HLA-A2 positive TAP deficient T2 cell lines T2-1B (HLA-A2/NY-ESO-1 157-165) and T2-1C (HLA-A2/NY-ESO-1 155-163) were cultured in R10 media along with 2.5 µg/ml hygromycine B(Held, Matsuo et al. 2004). All cells were cultured at 37 °C and 5 % CO2. 293T and MCF7 cell lines from culture flasks were detached by using PBS-1 mM EDTA. Cells were collected from the flask and centrifuged at 1500 rpm for 5 min. Cells were re-suspended in fresh culture medium. $10X10^6$ cells were frozen in 1.5 ml cryotube in 1 ml of freezing media (90 % FBS (v/v) 10% DMSO (v/v)).

3.19.2 Determination of cell number

Cell number of all cell lines and primary cells were determined by CASY cell counter+ analyser system model TT (Roche Innovatis, Bielefeld, Germany). 50 µl of sample cells were mixed with 10 ml of CASYton solution and analysed with the cell counter.

3.19.3 Mycoplasma test

Mycoplasma contamination was tested for every cell line using MycoAlert Mycoplasma detection Kit (Lonza Cologne GmbH, Cologne, Germany) in three months.

3.19.4 Purification of human CD4+ and CD8+ T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by density gradient centrifugation using Ficoll and PBMCs were washed with cold Phosphate-buffer saline (PBS) containing 0.5 % (w/v) FBS and 2 mM EDTA. CD4+ and CD8+ T cells were positively

selected using the CD4+ and CD8+ T cell isolation kits (Miltenyi Biotech, Germany) according to manufacturer's instruction.

3.19.5 Activation of T cells

Freshly isolated human T cells were stimulated with R10 media containing 100 ng/ml of anti-human CD3 (OKT3) and 100 ng/ml anti-human CD28 mouse monoclonal antibodies along with 400 IU of human recombinant IL-2.

3.19.6 Generation of re-directed $CD8^+$ anti-NY-ESO-1 T cells

Redirected T cells were generated as described previously. In short, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by density gradient centrifugation using Ficoll gradient. $CD8^+$ T cells were negatively selected using the $CD8^+$T cell isolation kit (Miltenyi Biotech, Germany) according to manufacturer's instructions. Anti-HLA-A*0201/NY-ESO-$1_{157–165}$ (anti-NY-ESO-1 CAR) scFv fragment, flanked by NcoI and BamHI restriction sites were cloned into the pBullet vector containing human CD3ζ and CD28 signaling domains. The BW431/26-CD28/CD3ζ CAR construct that directly recognizes the carcinoembryonic antigen (CEA) served as control and was termed anti-CEA CAR. The retroviral transduction of $CD8^+$ T cells with recombinant receptors was performed by co-culturing the polyclonally activated $CD8^+$ T cells with transiently transfected 293T cells as described. After 24h of co-cultivation, expression of recombinant receptors was monitored by flow cytometric analysis using PE-labeled anti-human IgG1, HLA-A*0201/NY-ESO-$1_{157–165}$ tetramer labeled with PE and FITC-conjugated anti human-CD8 purchased from Biolegend (San Diego, CA).

3.19.7 Peptide loading of T2 cells

The NY-ESO-1 $_{157-165}$ SLLMWITQV peptide (9v) or the influenza matrix protein (IMP) 58-66 GILGFVFTL peptides were dissolved in DMSO to obtain 10 µg/ml concentration. For the loading of peptide T2 cells were washed two times with serum free RPMI and 50,000 T2 cells were incubated with different concentration of peptides for 2 h at 37 °C. After one wash, cells were incubated with anti-NY-ESO-1 T1 CAR re-directed $CD8^+$ T cells.

3.19.8 Intracellular cytokine staining (ICS)

$2X10^5$ anti-NY-ESO-1 CAR re-directed $CD8^+$ T cells were incubated either with T2-1B cells, T2-1C cells or with medium alone (control) in the presence of 5 µg/ml Brefeldin A and 5 µg/ml monensin at 37 °C for 5 h. Cells were surface stained with anti-human CD8-PE-Texas Red and anti-human IgG-PE Southern Biotech (Birmingham, AL, USA) monoclonal antibodies for at 4 °C 15 min. After surface staining, cells were washed with FACS buffer (FB; PBS + 2 % FCS + 40

mM EDTA + 0.05 % NaN_3), fixed with 2% paraformaldehyde and permeabilized with permeabilization buffer (PB; FACS buffer + 0.1 % saponin). Cells were stained for intracellular IFNγ, TNFα and IL-2 with specific monoclonal antibodies (Biolegend, San Diego, USA) at 4 °C for 15 min. Samples were measured with a CyAn ADP9 flow cytometer (Beckman Coulter, Brea, CA, USA) and results were analyzed using FlowJo analysis software.

3.19.9 Colorimetric analysis of cell cytotoxicity

$CD8^+$T cells were co-cultured in 96-well round bottom microtiter plates at different numbers (ranging from 2,500-20,000 CAR-positive T cells per well) with 10,000 HLA-A2/NY-ESO-$1_{157–165}$-positive cells (T2-1B) or control cells in 200 µl of R10 medium. After 24 h, XTT reagent (Cell Proliferation Kit II, Roche Diagnostics, Rotkreuz, Switzerland) was added to the cells and incubated at 37 °C for 30–90 min. Reduction of XTT to formazan by viable tumor cells was colorimetrically monitored. Maximal reduction of XTT was determined as the mean of 3 wells containing target cells only, and the background as the mean of 3 wells containing R10 medium. The non-specific formation of formazan due to the presence of effector cells was determined from triplicate wells containing effector cells in the same number as in the corresponding experimental wells.

3.19.10 5-aza-2’ Deoxycytidine (DAC) treatment

MCF7 (breast cancer) and U266 (multiple myeloma) cells were cultured in R10 medium. $1X10^6$ MCF7 and U266 Cells were plated in T25 culture flasks, followed by treatment with R10 media containing 10 µM of 5-aza-2’ Deoxycytidine (DAC) after 24 h, for four times in a day, Control group was cultivated in the absence of DAC. DAC treatment was carried for three days and cells were cultivated in R10 medium (without DAC) for additional two days. Cells were then subjected to cytotoxic assay and IFNγ ELISA by co-culturing with anti-NY-ESO-1 T1 CAR re-directed CD8+ T cells.

3.19.11 Binding of anti-idiotypic Fab antibodies with anti-NY-ESO-$1_{155–163}$/HLA-A*0201 antibody

A4 and H6 anti-idiotypic Fabs were coated on the surface of 96-well ELISA (Nunc, Langenselbold, Germany) plates at a concentration of 0.01 µg/µl in carbonate buffer (pH 9.5) at 37 °C for 3-4 h. Unbound Fabs were removed by PBS (pH 7.4) washing. Wells were subsequently blocked with PBS plus 10 % FCS for 1 h at room temperature (RT). Anti- NY-ESO-$1_{155–163}$/HLA-A*0201 (3M4E5) or control hIgG antibodies at a concentration of 1 ng/µl were added to the anti-idiotypic antibody coated wells and incubated for 1 h at RT. Plates were

washed three times with PBS containing 0.05 % Tween-20 to remove unbound antibodies. Binding of the 3M4E5 antibody was accessed with HRP-labeled anti-human Fc specific antibody (ImmunoResearch, Suffolk, Great Britain) according to the manufacturer's instructions.

3.19.12 Surface Binding of Anti-Idiotypic Fab antibodies

293T cells were transfected with the anti-NY-ESO-1 CARs as described previously (Schuberth, Jakka et al. 2012). After 24 h, 10^5 anti-NY-ESO-1 CAR grafted 293T cells were washed twice with PBS (pH 7.5) containing 2 mM EDTA and 0.05 % FCS (FACS buffer). Cells were re-suspended in 0.01 µg/ul anti-idiotypic Fabs in FACS buffer and incubated for 30 min at 4 °C. Cells were washed with FACS buffer and incubated with mouse anti-His tag monoclonal antibody (Qiagen, Hombrechtikon, Switzerland) for 30 min. Followed by PE-labeled anti-mouse IgG1 antibody (Southern Biotech, Birmingham, AL). Binding of anti-idiotypic Fabs was analyzed by FACScan (BD Bioscience, San Diego, CA). Data were analyzed using FlowJo software (Tree Star, Asland, OR).

3.19.13 Competition assay of binding of anti-idiotypic Fab molecules

10^5 anti-NY-ESO-1 CAR $CD8^+$ T cells were washed twice with FACS buffer. Anti-NY-ESO-1 CAR re-directed $CD8^+$ T cells were incubated with different concentrations of anti-idiotypic Fab A4 (1, 0.25, 0.03 µg/µl) for 15 min at RT, 1 µg/µl of irrelevant Fab served as control for anti-idiotypic Fab. 10 µl of PE-labeled HLA-A*02:01/NY-ESO-$1_{157\text{-}165}$ tetramer (0.002 µg/µl) was added to each sample. Tetramer alone served as a negative control. Tubes were incubated for 5 min at RT. Cells were washed with FACS buffer and inhibition of tetramer binding was determined by fluorescent intensity measured by FACScan (BD Bioscience, San Diego, CA). Data were analyzed using FlowJo software (Tree Star, Asland, OR).

3.19.14 Activation of anti-NY-ESO-1 CAR re-directed $CD8^+$ T cells with anti-idiotypic or with HLA-A2 dimer pulsed with NY-ESO-$1_{157\text{-}165}$ peptide

2 to 200 nM anti-idiotypic Fab antibody or recombinant HLA-A2 dimer (BD Bioscience, San Diego, CA) were coated on 96 well cell culture plates at 37 °C for 3 h or at 4 °C overnight. Plates were washed with PBS to remove unbound molecules. 100 µl of 10 µM NY-ESO-$1_{157\text{-}165}$ peptide was added to the recombinant HLA-A2 coated wells that were subsequently incubated at 37 °C for 3 h. Plates were washed with PBS; 2 x 10^4 anti-NY-ESO-1 CAR re-directed $CD8^+$ T cells were added to each well. After 24 h of stimulation, secretion of IFNγ was measured using an IFNγ ELISA kit (BD OptEIATM, San Diego, CA) according to the manufacturer's instructions.

3.19.15 Antigen-dependent expansion of anti-NY-ESO-1 CAR re-directed CD8$^+$ T cells

25 cm^2 tissue culture flasks were coated with 2 µg/ml of anti-NY-ESO-1 CAR specific anti-idiotypic Fab antibodies or control Fab (irrelevant Fab) molecules and incubated at 37 °C for 3-4 h. 10^6 anti-NY-ESO-1 CAR positive re-directed CD8$^+$ T cells were added to the flasks in 5ml of RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (R10 medium) and 50 IU/ml of human recombinant (rh) IL-2. The medium was replaced with fresh R10 medium containing rh IL-2 (50 IU/ml) after 24 h of stimulation. For T2-1B mediated activation, 2.5x10^5 irradiated (γ-irradiation with 50 Gy) stimulator cells (T2-1B) were co-cultured as described above at a ratio of 4 to 1 (effector to target). Re-directed T cells were stimulated repetitively with irradiated T2-1B cells or anti-idiotype antibody every 8 days. Increase in receptor positive cells was identified by surface staining with anti-human IgG antibody and the cell number was determined by counting viable cells on a weekly basis.

3.19.16 Phenotyping of expanded re-directed CD8+ T cells

Phenotype analysis of expanded anti-NY-ESO-1 T1 CAR redirected CD8+ T cells 0.5 x 10^6 redirected T cells were washed with FACS buffer and surface stained with anti-human IgG-PE, CD8-PE-Cy7, CCR7-FITC and CD62L-APC; all the antibodies were used according to manufacturer's instructions. Cells were washed and fixed with 2 % paraformaldehyde for 10 min at RT. After washing cells were re-suspended in FACS buffer and analyzed by flow cytometer. Flow cytometry analysis was performed using FACSCanto II.

3.19.17 IgE ELISA

The anti-tumor effect of the expanded anti-NY-ESO-1 T1 CAR re-directed CD8+ T cells was assessed by measuring tumor size and serum IgE levels. Blood was collected from mouse tail vein and serum was separated for measuring IgE protein levels. Maxisorp 96-well flat bottom plates (NUNC, Rochester, USA) were coated with 5 µg/ ml (100 µl/well) purified anti-human IgE antibody diluted in carbonate buffer (pH 9.6) and incubated at 3-4 h at 37 °C. Unbound antibodies were removed by washing plates with washing buffer (PBS/0.05 % tween 20) and blocked with PBS containing 10 % FBS at room temperature (RT) for 1 h. After washing (three times), mouse serum samples and standard (purified human IgE protein) were added to the wells and incubated at RT for 1 h. Plates were washed 5 times and then incubated with 1 µg/ml of biotinylated anti-human IgG ,F(ab)2 specific antibody diluted in 10 % FBS/PBS for 1 h at RT. Plates were washed five times and 1 µg/ml of streptavidin-Horse Radish Peroxidase was added (1h at RT). Substrate was added after 5 times of washing and reaction was stopped using 2N H2SO4 (Sigma-Aldrich, St.Louis, USA) and measured at 450 nm.

3.19.18 Xenograft model

In vivo anti-tumor functional studies: NOD-SCIDγc (null) (NSG) mice were bred and maintained under specific pathogen free conditions in house. Six to eight week old NSG mice were injected subcutaneously with $10X10^6$ multiple myeloma U266 tumor cells with or without expanded anti-NY-ESO-1 T1 CAR re-directed CD8+ T cells. Tumor growth was assessed by measuring serum IgE levels every week and tumor size was measured with the help of caliper in two perpendicular dimensions and the tumor volume was calculated using the formula: Tumor size in mm2=(length X width).

4 Results

4.1 Re-directed T cells

Previously, our group has selected a TCR like antibody (3M4E5) from a large phage display library that recognizes the HLA-A2/NY-ESO-1 $_{157\text{-}165}$ peptide (Held, Matsuo et al. 2004). Selective mutation in the peptide binding region of two light chain amino acids of the 3M4E5 Fab molecule enhanced the affinity by 20 fold (3M4E5-T1) in the range of 2-4 nM. In our group, chimeric antigen receptors (CARs) were constructed from the scFv of 3M4E5 (anti-NY-ESO-1 CAR) and the 3M4E5-T1 (anti-NY-ESO-1 CAR T1) Fab molecules (Stewart-Jones, Wadle et al. 2009). ScFvs were cloned into the optimized pBULLET retroviral vector in line with the CH2/3 IgG domain and the CD28-CD3ζ domains (Hombach, Wieczarkowiecz et al. 2001). CD8+ T cells were positively selected from human PBMCs isolated from buffy coats to express the chimeric antigen receptors. For the generation of CAR gene carrying retrovirus, 293T cells were transiently transfected with the specific CAR expressing cassette along with two helper plasmids, one for the *gag* and *pol* protein of the retrovirus and the other one for *env* protein. To express the CARs in CD8+T cells, activated CD8+ T cells were co-cultured with transiently transfected 293 T cells. CARs were expressed in CD8+ T cells consisting of scFv derived from 3M4E5 (anti-NY-ESO-1 CAR) and 3M4E5-T1 (anti-NY-ESO-1 CAR T1) linked to CH2/3 IgG-CD28-CD3 ζ domains. Anti-CEA CAR (BW431/26-CD28/CD3ζ) re-directed CD8+ T cells were used as control (construct kindly provided by Prof. Abken) (Chmielewski, Hahn et al. 2012). The schematic representation of CAR constructs is shown in figure 1.

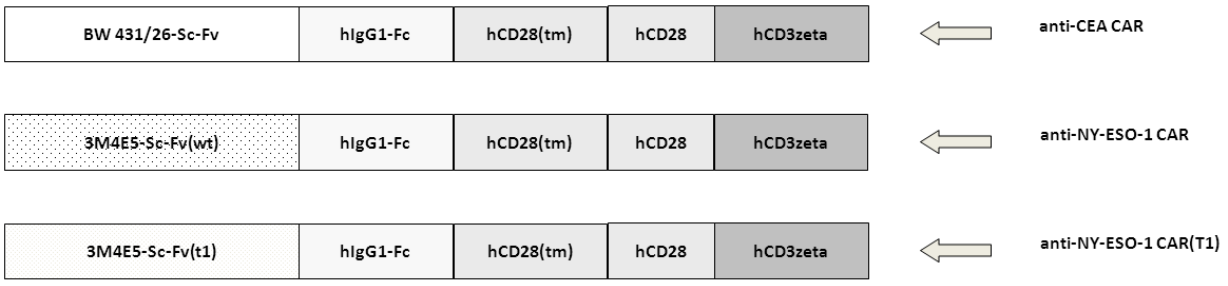

Figure 4-1: Schematic representation of expression cassettes for the recombinant anti-NY-ESO-1 CARs and control immuno receptor anti-CEA CAR.

4.2 Transduction

Expression of CARs on CD8+ T cell surface was assessed by surface staining of human immunoglobulin CH2/CH3 domains with anti-human IgG Fc specific mouse monoclonal antibodies using flow cytometry. 28-50 % of CD8+ T cells expressed the respective CAR on their cell surface (figure 2A). Antigen specific binding of anti-NY-ESO-1 CARs were measured by using a HLA-A2/NY-ESO-1 $_{157\text{-}165}$ peptide tetramer-PE conjugate. Similar results were observed with tetramer staining as detected with anti-human IgG Fc specific antibody; whereas no binding of tetramer was observed in anti-CEA CAR expressing CD8+ T cells (figure 2B). This observation shows that surface expressed anti-NY-ESO-1 CARs specifically bind to the NY-ESO-1 157-165 peptide in context with HLA-A2 molecule (Schuberth, Jakka et al. 2012).

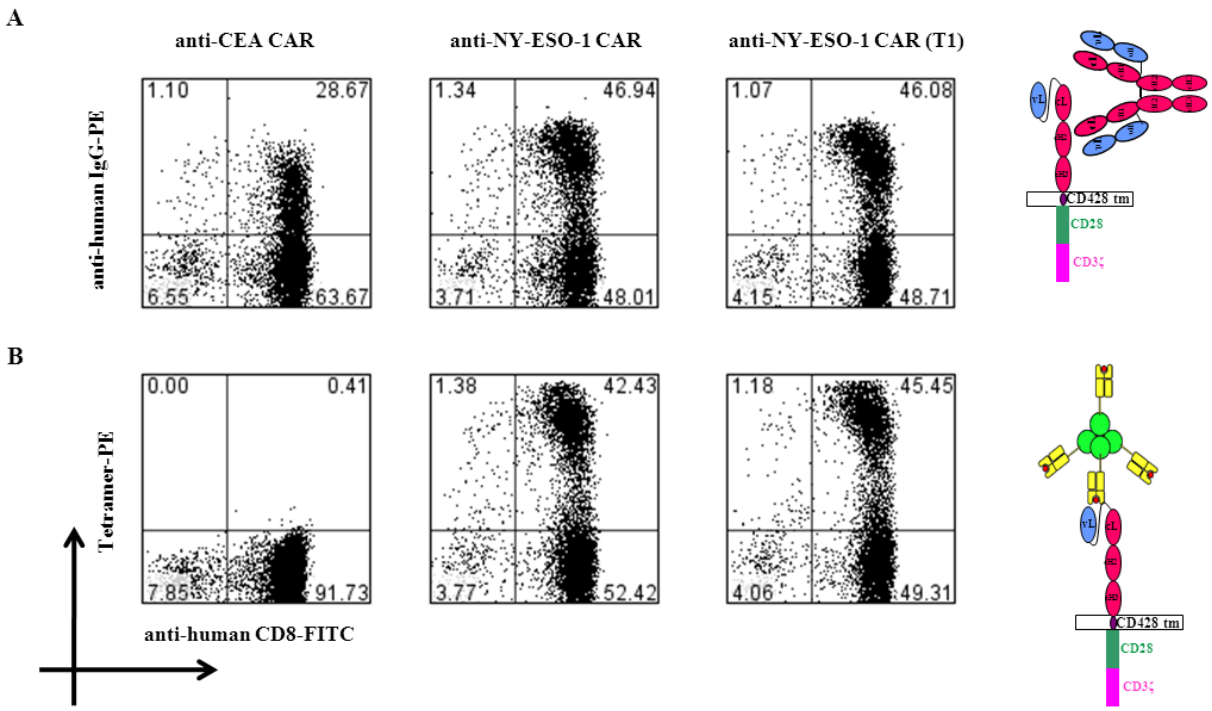

Figure 4-2: A surface expression of the grafted receptors on CD8+ T cells confirmed by flow cytometry analysis. Transduced CD8+ T cells were simultaneously incubated with PE-conjugated anti-human IgG1 and FITC-conjugated anti-CD8 mAb. **B** Antigen specific binding confirmed by NY-ESO-1$_{157\text{-}165}$/HLA-A2-teramer-PE and FITC-conjugated anti-CD8 mAb.

4.3 Characterization of target cells

TAP deficient T2 cells were used to test for antigen-specific functionality of re-directed CD8 T cells. These cells express low amounts of MHC class 1 on the cell surface. Transfection of these T2 cells with minigenes encoding HLA-A2 peptide linked to ER signaling sequences

enhances the stable expression of HLA-A2 molecules on the cell surface (Zweerink, Gammon et al. 1993). For the stable expression of NY-ESO-1 peptides, T2 cells were transfected with minigenes, expressing the NY-ESO-1 157-165 peptide (T2-1B) or NY-ESO-1 155-163 (T2-1C), respectively. We analyzed the surface expression levels of HLA-A2 molecules on minigene transfected T2 cells. Results revealed similar levels of HLA-A2 expression on both peptide transfected T2 cells (Figure 3A). Next, we confirmed the expression of NY-ESO-1 157-165 peptide in the context of HLA-A2 on the cell surface of transfected T2 cells using anti-NY-ESO-1 (T1) Fab tetramer-PE, which is specific for HLA-A2/NY-ESO-1 $_{157\text{-}165}$. We observed the specific binding of anti-NY-ESO-1 tetramers with T2-1B cells and no specific signal was observed with T2-1C cells (figure 3B). Furthermore, we analyzed the HLA-A2 and NY-ESO-1 expression on tumor cell lines: The multiple myeloma cell line U266 is known to be positive for HLA-A2 and expresses NY-ESO-1 protein. The breast cancer cell line MCF7 expresses similar levels of HLA-A2 on the cell surface and does not express NY-ESO-1 protein (figure 3C). These results were further confirmed by Western blot (figure 3D). With these different cell lines, antigen-specific functionality could be tested in further experiments.

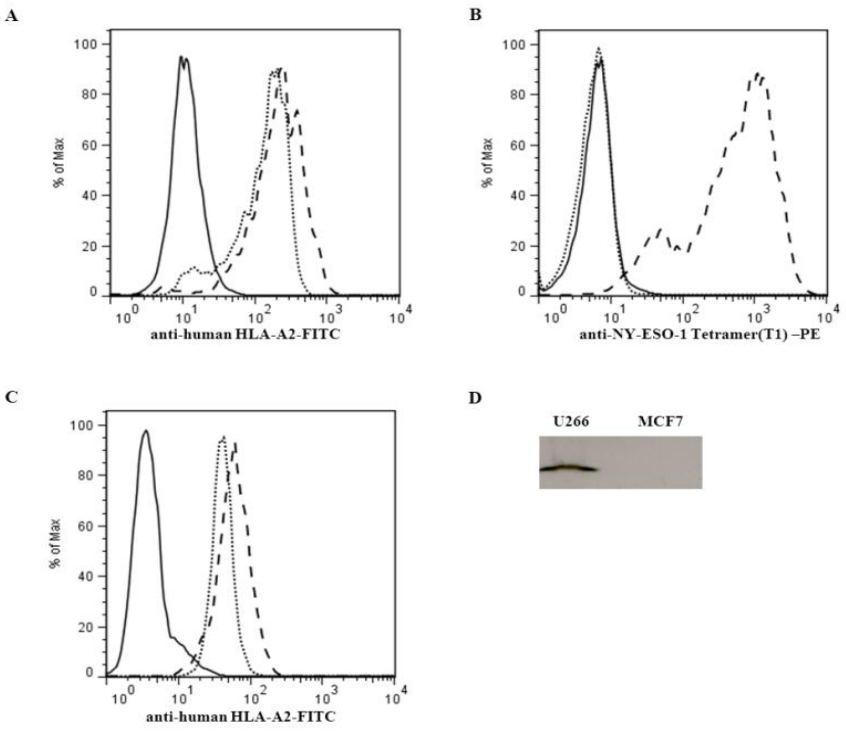

Figure 4-3: Surface staining of minigene transfected T2 cells**. A** Surface expression of HLA-A2 was determined by staining of T2-1B (dashed line) and T2-1C (dotted line) with anti-human HLA-A2 FITC and isotype control antibodies. B Surface expression of NY-ESO-1 peptide in context of HLA-A2 was determined by staining with anti-HLA-A2/NY-ESO-1 $1_{57\text{-}165}$ tetramer-PE (T1). **B** T2-1B (dashed line) and

T2-1C (dotted line) cells were stained with anti-HLA-A2/NY-ESO-$1_{157-165}$ tetramer-PE(T1). **C** Expression of HLA-A2 and NY-ESO-1 protein in U266 multiple myeloma cell line (dashed line) and MCF7 breast cancer cell line (dotted line) was confirmed with anti-human HLA-A2 –FITC staining .D Western blot analysis of NY-ESO-1 protein expression in U266 and MCF7 cells.

4.4 Characterization of re-directed T cells

4.4.1 Polyfunctional analysis of re-directed T cells

With help of the characterized cell lines, antigen-specific poly-functionality of the re-directed T cells was evaluated by intracellular cytokine staining. We co-incubated freshly transduced re-directed anti-NY-ESO-1 CAR and anti-NY-ESO-1 CAR (T1) positive CD8+ T cells with T2-1B (antigen specific) T2-1C (control) for 4 hours. As shown in figure 4A, both CARs re-directed CD8+ T cells in a poly-functional manner. T cells secreted the cytokines IFNγ, TNFα and IL2 in an antigen specific manner. In contrast, no to basal activation was observed with control cells. We observed significant difference in cytokine production between antigen specific and control cell activation (figure B).

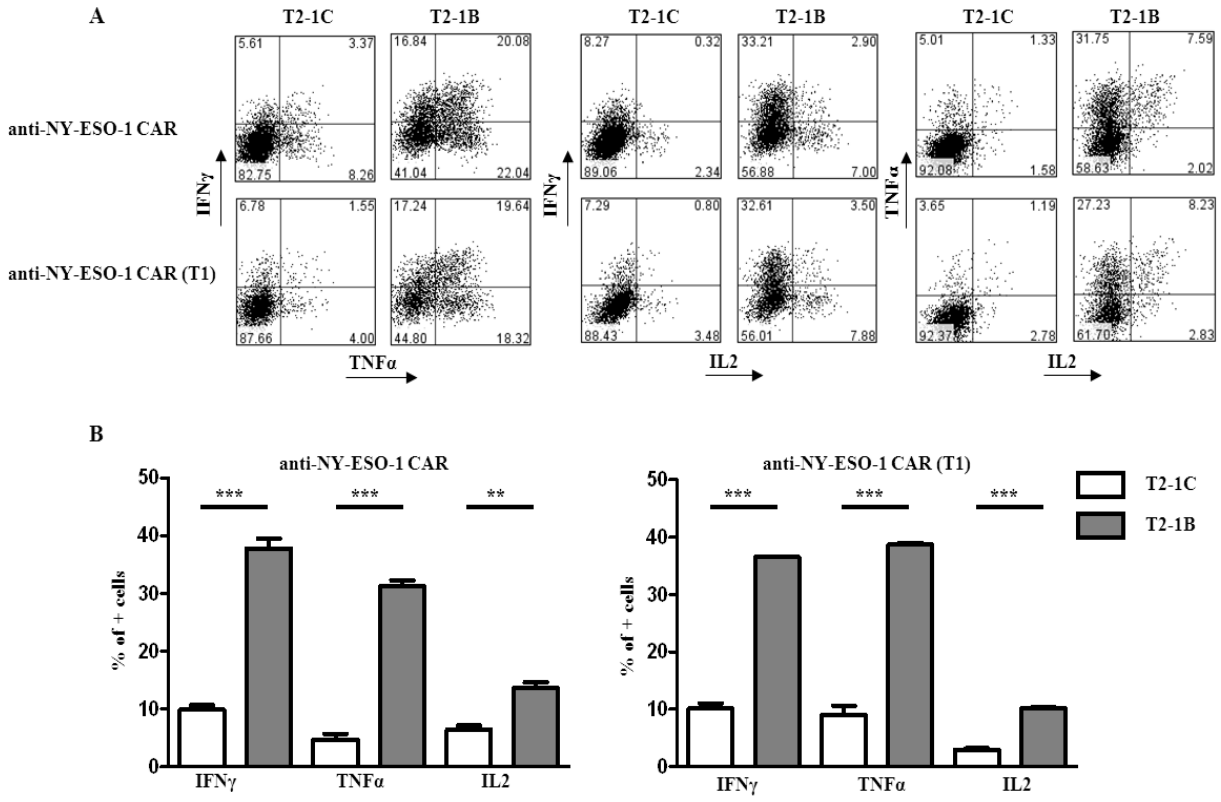

Figure 4-4: Poly-functional analysis of CAR re-directed CD8+ T cells. Antigen specific cytokine secretion of anti-NY-ESO-1 CARs was determined by intracellular staining. Anti-NY-ESO-1 CAR and anti-NY-ESO-1 CAR(T1) re-directed CD8+ T cells were incubated with T2-1B and T2-IC cells for 4 hours . A: dotplot

indicating percentage of IFNγ, TNFα and IL2 positive cells. B: Statistic analysis of N=3 experiments of cells secreting IFNγ, TNFα and IL2.

4.4.2 Anti-NY-ESO-1 CAR expressing re-directed CD8+ T cells lysed the antigen expressing target cells and secreted cytokines.

Since we demonstrated antigen-specific cytokine secretion of CD8 positive re-directed T cells, we aimed to demonstrate effector cell function. In order to demonstrate functional differences in the lysis between anti-NY-ESO-1 CAR and anti-NY-ESO-1 CAR (T1)**,** we performed XTT based colorimetric cytotoxic assay to assess target cell lysis of NY-ESO-1 157-165 peptide expressing T2-1B cells as target and NY-ESO-1 155-163 peptide expressing T2-1C as control cells. We observed a significant increase in lysis of target cells (T2-1B) with anti-NY-ESO-1 CARs re-directed CD8+ T cells when compared with control anti-CEA CAR re-directed T cells (figure 5A). We have not observed any significant lysis of T2-1C cells with any of the re-directed T cells (figure 5B). Furthermore, we observed a significant difference between wild type anti-NY-ESO-1 CAR and affinity matured anti-NY-ESO-1 CAR (T1) at lower effector target ratios, which was not observed at higher effector target ratios (figure 5A). T cell activation marker IFNγ and granzyme B were measured in supernatants of these experiments. IFNγ and granzyme B secretion was observed specifically with T2-1B culture supernatants (figure 5C&E) in antigen specific manner. Only background levels were observed with T2-1C control cells and control anti-CEA CAR (figure 5D&F).

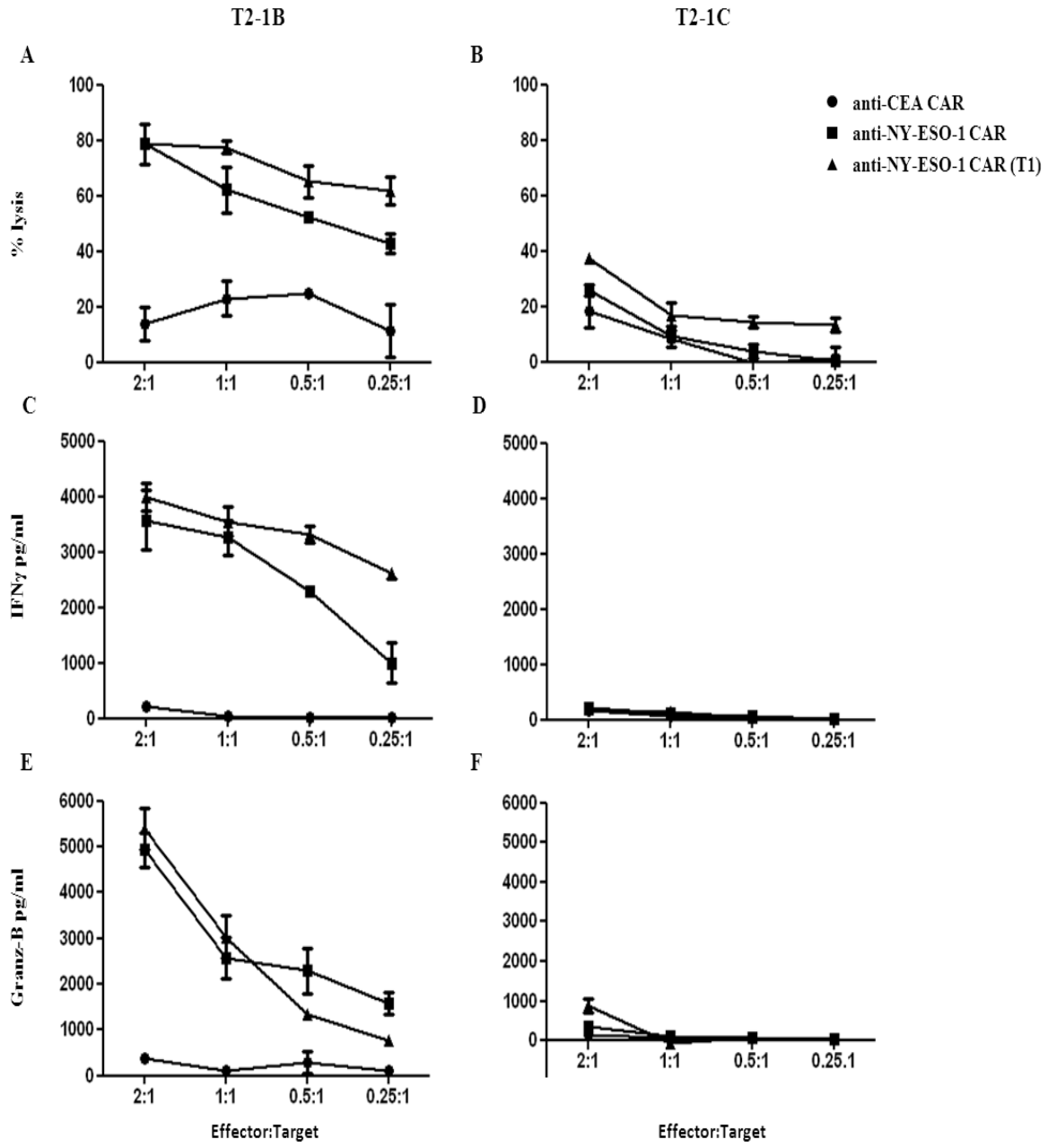

Figure 4-5: Comparison of anti-NY-ESO-1 wild type and affinity matured (T1) chimeric antigen receptor re-directed CD8+ T cells. **A&B** anti-NY-ESO-1 CAR, anti-NY-ESO-1 CAR (T1) and anti-CEA CAR re-directed CD8+ T cells were co-cultivated with minigene transfected T2 (TAP-deficient) cell lines T2-1B and T2-1C for 24h. T2-1B expressed NY-ESO-$1_{157-165}$ /HLA-A2, whereas T2-1C expresses NY-ESO-$1_{155-163}$ /HLA-A2. Transduced cell populations were adjusted to obtain equal effector cell numbers. Viability of tumor cells was determined colorimetrically by a tetrazolium salt-based XTT assay after 24h. **C&D** IFN-gamma and **E&F** granzyme B secretion by CAR re-directed CD8+ T cells into the culture supernatant was determined by ELISA.

4.4.3 Anti-NY-ESO-1 TCR CAR activates both CD4+ and CD8+ T cells

MHC restricted and co-receptor independent function of anti-NY-ESO-1 CAR (T1) was evaluated in purified populations of CD4+ and CD8+ T cells. The anti-NY-ESO-1 CAR (T1) and anti-CEA CAR re-directed CD4+ and CD8+ T cells were co-cultured with T2-1B and T2-1C cells for 24 h. Antigen specific IFNγ secretion was observed in both, CD4+ and CD8+ T cell populations and only background levels of cytokine secretion were observed with anti-CEA CAR and T2-1C cells (figure 6A&B), respectively. These results suggest that CARs generated from TCR like antibodies function in a MHC-restricted and co-receptor independent manner. Furthermore, the sensitivity of anti-NY-ESO-1 CAR (T1) re-directed T cells was analyzed by co-culturing the re-directed CD4+ and CD8+ T cells with peptide pulsed T2 cells. IFNγ secretion was enhanced in a dose dependent manner in anti-NY-ESO-1 CAR (T1) re-directed CD4+ and CD8+ T cells whereas no IFNγ secretion was observed with control peptide (Flu) pulsed T2 cells and with anti-CEA CAR re-directed T cells. These results indicate that increase in peptide density on cell surface increases T cell function (figure 6 C& D).

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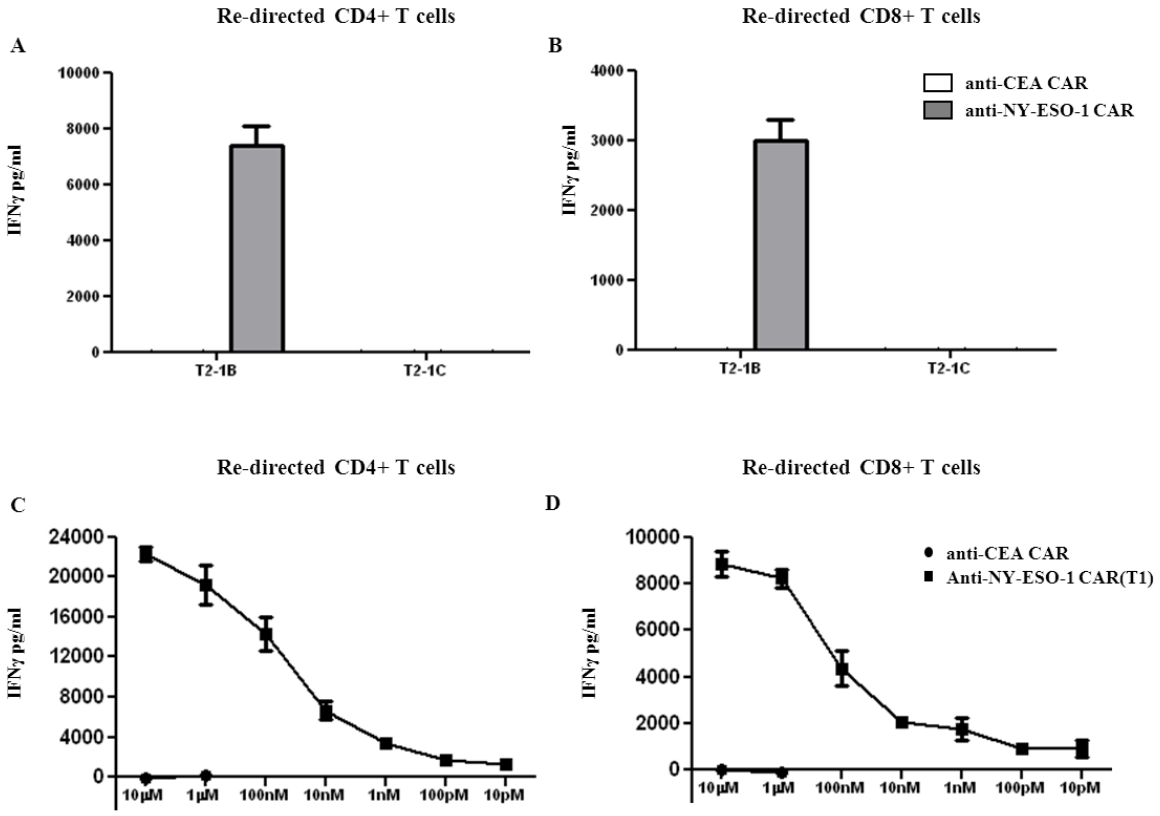


Figure 4-6: Anti-NY-ESO-1 CAR generated from TCR like antibody functions in re-directed CD4+ and CD8+ T cells in MHC restricted and co-receptor independent manner. Purified human CD4+ and CD8+

T cells were re-directed with anti-NY-ESO-1 CAR (T1). **A&B** CD4+ and CD8+ T cell populations were co-incubated with T2-1B and T2-IC cells for 24 hours. T cell activation marker IFNγ was measured in culture supernatant. **C&D** T2 cells were pulsed with different concentration of NY-ESO-1 157-165 peptide and incubated with anti-NY-ESO-1 CAR(T1) re-directed CD4+&CD8+ T cells for 24 hours. IFNγ was measured from culture supernatants.

4.4.4 5-aza-2-deoxycytidine (DAC) treatment enhances anti HLA-A2/NY-ESO-$1_{157–165}$ peptide specific re-directed CD8+ T cell effector function.

In the previous experiment we have demonstrated that increased HLA-A2/NY-ESO-$1_{157–165}$ peptide-load augments killing of target cells (Figure 5-5). Therefore, we tested whether DAC treatment enhanced the anti-NY-ESO-1 CAR (T1) activity by increased peptide expression. We transduced CD8+ T cells with anti-NY-ESO-1 CAR (T1) and anti-CEA CAR as a control. DAC treated MCF7 cells specifically activated anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells only whereas no activation was observed with untreated MCF7 cells. The Multiple myeloma cell line U266 that constitutively expresses NY-ESO-1 protein were lysed even in the absence of DAC treatment. Moreover, DAC treatment enhanced the lysis significantly. No specific lysis was observed with control CAR re-directed T cell (figure7 A). We measured the T cell activation marker IFNγ in the culture supernatant of both DAC treated and untreated samples, specific IFNγ secretion was observed with DAC treated MCF7 cells and U266 cells, where IFNγ secretion was significantly increased in DAC treated U266 cells (figure7 B). These results indicate that increasing peptide density on cell surface with DAC-treatment enhanced the antigen specific function of re-directed T cells.

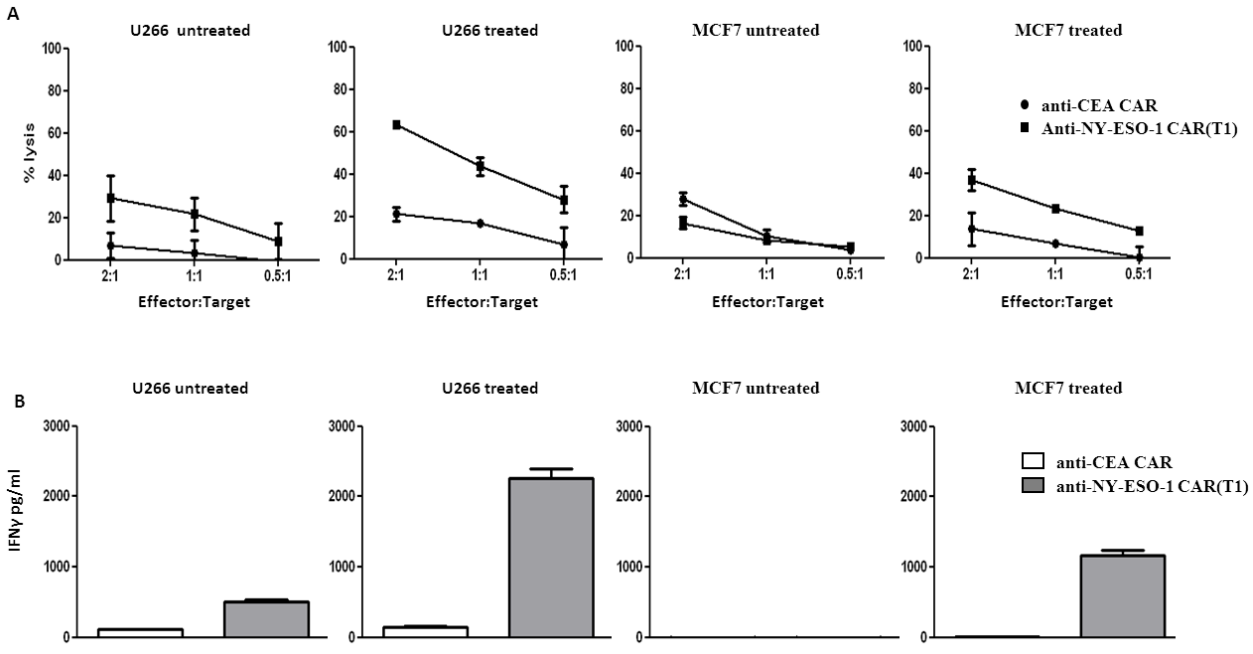


Figure 4-7: A. Treatment of the NY-ESO-1 positive multiple myeloma cell line (U266) and NY-ESO-1 negative breast cancer cell line MCF7 with the demethylating agent 5-aza-2-deoxycytidineincreases (DAC). NY-ESO-1 expression thus enhancing the killing by NY-ESO-$1_{157\text{-}165}$ specific anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells.**B.** Specific activation of anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cellswas determined by measuring the IFN-gamma secretion in culture supernatant by ELISA.

4.5 Expansion of re-directed T cells

4.5.1 Selection and characterization of anti-idiotypic Fab molecules for antibody recognizing HLA-A*0201/NY-ESO-$1_{157–165}$ (anti-NY-ESO-1)

Since we showed that re-directed T cells were antigen-specifically activated by cells expressing the HLA-A*0201/NY-ESO-$1_{157–165}$ complex we questioned whether anti-idiotypic antibodies also would activate re-directed T cells. Therefore, anti-idiotypic scFvs against the 3M4E5 CAR (recognizing HLA-A*0201/NY-ESO-$1_{157–165}$) were selected from phage display library as described previously (Held, Matsuo et al. 2004). Two antibodies - A4 and H6 - which differ in the sequence of the variable domain were selected (data not shown). A4 and H6 scFv were cloned into bacterial expression vectors. After induction, both anti-idiotypic Fab molecules were expressed with an expected size of 25 kD (for each heavy and light chain) (figure 8A). Both, A4 and H6 bound to anti-NY-ESO-1 antibody but not to the control antibodies (anti-VEGF or anti-CD20 mAB) as analyzed by ELISA. Furthermore, A4 and H6 had similar binding properties to the anti-NY-ESO-1 antibody (figure 8B).

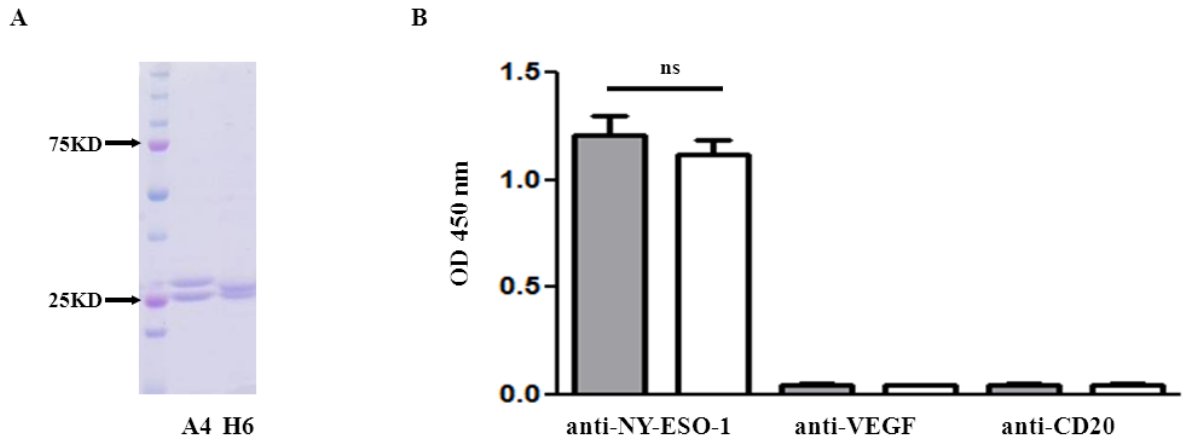


Figure 4-8: Expression and purification of soluble anti-idiotypic Fab molecules (A4 and H6) binding to 3M4E5 antibody recognizes the NY-ESO-$1_{157\text{-}165}$ peptide in the context of HLA A*0201.**A** Two different purified anti-idiotypic Fab molecules on SDS PAGE gel (A4 and H6). **B** Binding of soluble purified anti-idiotypic Fab molecules A4 (grey) and H6 (white) to immobilized anti HLA-A0201/NY-ESO-$1_{157–165}$

(3M4E5) molecules was determined by ELISA. Anti-CD20 and anti-VEGF antibodies were used as control. ***P<0.0003

4.5.2 Anti-idiotypic Fab A4 binds to cell surface expressed anti-NY-ESO-1 CAR (T1)

To confirm that anti-idiotypic Fab A4 recognizes the anti-NY-ESO-1 CAR when expressed on the cell surface, anti-NY-ESO-1 CAR (T1) and anti-CEA CAR transfected 293T cells were used (75 to 90 % CAR expression; Figure 9A). As expected, anti-idiotypic Fab A4 as HLA-A*0201/NY-ESO-$1_{157–165}$ tetramer bound to anti-NY-ESO-1 CAR (T1) only (Figure 9B). A4 and A*0201/NY-ESO-$1_{157–165}$ tetramer did not bind to control anti-CEA CAR.

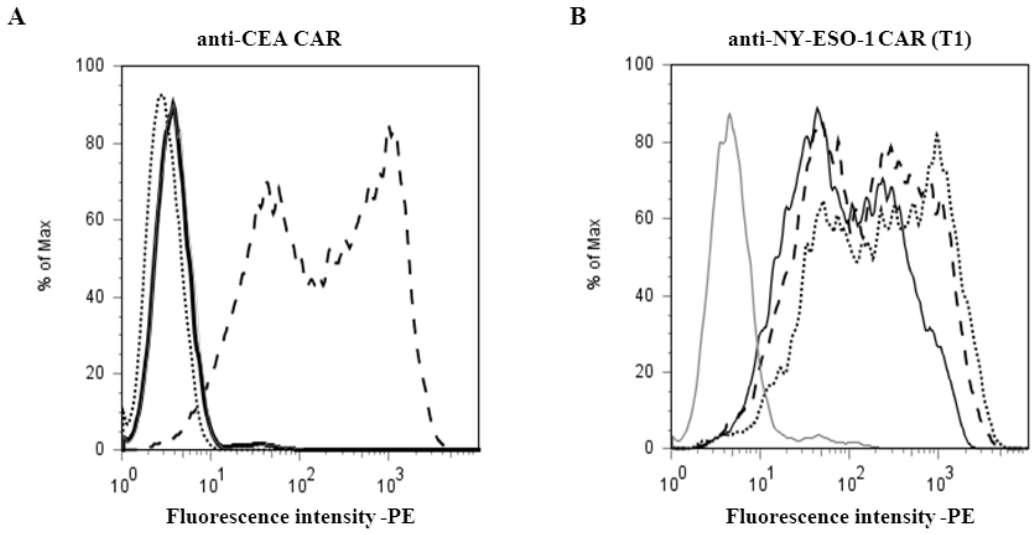


Figure 4-9: Surface binding of soluble purified Fabs to anti-NY-ESO-1 CAR (T1) transfected 293T cells. 293T cells were transfected with anti-CEA CAR and anti-NY-ESO-1 CAR (T1). **A** Expression of both CARs was determined by staining with anti-human IgG antibody (dotted line).**B** Correct folding of anti-NY-ESO-1 CAR was determined by staining with HLA-A*0201/NY-ESO-$1_{157–165}$ tetramer-PE (dashed line). Fab (A4) bound to anti-NY-ESO-1 CAR (T1) (black line). As negative control, transfected 293T cells stained with irrelevant control Fab molecule (light grey).

4.5.3 Affinity measurement of anti-idiotypic Fab A4 and competition with HLA-A*0201/NY-ESO-$1_{157–165}$tetramer

Apparent affinity constant (KDapp) of the anti-idiotypic Fab A4 was measured on anti-NY-ESO-1 CAR(T1) transfected 293T cells by analyzing the binding of serially diluted anti-idiotypic Fab A4 (Figure 10A). Half maximum fluorescence intensity was used to calculate the apparent affinity. Binding affinity of the anti-idiotypic Fab A4 to anti-NY-ESO-1 CAR(T1) was calculated as shown by KDapp values of 200 nM, which is comparable with data obtained from surface

Plasmon Resonance (SPR) on a CMS5 chip coated with anti-NY-ESO-1 Fab (data not shown). Next we analyzed the binding competition of anti-idiotypic Fab molecules with HLA-A*0201/NY-ESO-$1_{157–165}$ tetramer for anti-NY-ESO-1 CAR (T1), which was expressed on $CD8^+$ T cells. Anti-NY-ESO-1 CAR re-directed $CD8^+$ T cells were incubated with decreasing concentrations of anti-idiotypic Fab A4 (1, 0.25, 0.03 µg/µl) and competed with known concentration of HLA-A*0201/NY-ESO-$1_{157–165}$ tetramer (0.002 µg/µl), 1 µg/µl of irrelevant Fab served as control for anti-idiotypic Fab. Inhibition of tetramer binding was determined by FACS analysis. HLA-A*0201/NY-ESO-$1_{157–165}$ tetramer binding was inhibited in a dose dependent manner as shown in figure 10B. These results indicate that anti-idiotypic Fab A4 competes with HLA-A*0201/NY-ESO-$1_{157–165}$ tetramer for anti-NY-ESO-1 CAR (T1) and completely blocks the binding of tetramer with anti-NY-ESO-1 CAR (T1) at high concentration of anti-idiotypic Fab A4.

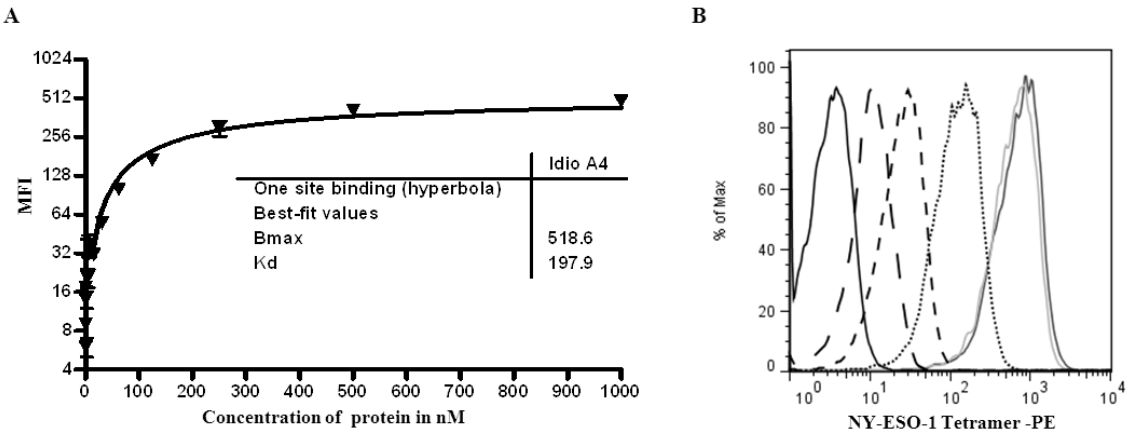


Figure 4-10: A For affinity studies, anti-idiotypic Fab molecules at various concentrations were incubated with NY-ESO-1 CAR transfected 293T cells. Detection was carried out with Penta-His, anti-mouse IgG (H+L) biotin, and streptavidin PE-antibody. For determination of apparent affinity of anti idiotypic Fab to anti-NY-ESO-1 Fab, half maximal binding concentration (KD) was calculated. **B** Competition between anti-NY-ESO-1 anti-idiotypic Fab molecules and HLA-A*0201/NY-ESO-$1_{157–165}$ tetramer was analysed. Competition assay was performed with anti-NY-ESO-1 CAR (T1) re-directed $CD8^+$ T cells. Re-directed T cells were saturated with different concentrations of anti-idiotypic Fab A4 (wide dashed: 1 ug/ul, dashed: 0.25 ug/ul, spotted: 0.03 ug/ul) and control Fab molecules (light grey: 1 ug/ul). After 15 minutes of incubation HLA-A*0201/NY-ESO-$1_{157–165}$ tetramer (0.002 ug/ul, dark grey: tetramer only) was added to the cells. Competition was measured by flow cytometry.

4.5.4 Anti-idiotypic Fab A4 activates anti-NY-ESO-1 CAR (T1) re-directed CD8$^+$T cells *in vitro*

Purified human CD8+ T cells were transduced with anti-NY-ESO-1 CAR (T1) and anti-CEA CAR by retrovirus-mediated gene transfer. To address anti-idiotypic Fab dependent specific activation of anti-NY-ESO-1 CAR (T1) re-directed CD8+T cells IFNγ secretion was measured by ELISA. Cells were stimulated by plate-bound anti-idiotypic or control Fab. Anti-NY-ESO-1 CAR(T1) grafted CD8+ T cells specifically secreted IFNγ in response to anti-idiotypic Fab A4, whereas no specific activation was observed in control Fab coated wells (figure 3A). Secretion of IFNγ was specific since only background levels of IFNγ were found in supernatants from anti-CEA CAR re-directed CD8+ T cells (figure 11A). Next we compared the activation potential of the anti-idiotypic Fab A4 molecules and recombinant human HLA-A2 molecules pulsed with NY-ESO-$1_{157\text{-}165}$ peptide. Equimolar concentrations of both molecules were immobilized to cell culture plate for comparison. IFNγ secretion was measured by ELISA from the supernatants. Anti-NY-ESO-1 CAR (T1) re-directedCD8+ T cells secreted IFNγ through activation of solid phase bound anti-idiotypic Fab A4 and recombinant human HLA-A2 molecules in a dose dependent manner (figure 11B). Anti-idiotypic Fab A4 revealed significantly higher levels of IFNγ at 66 nM and 22 nM, when compared with human HLA-A2 molecules pulsed with NY-ESO-$1_{157\text{-}165}$ peptide (Figure11 B).

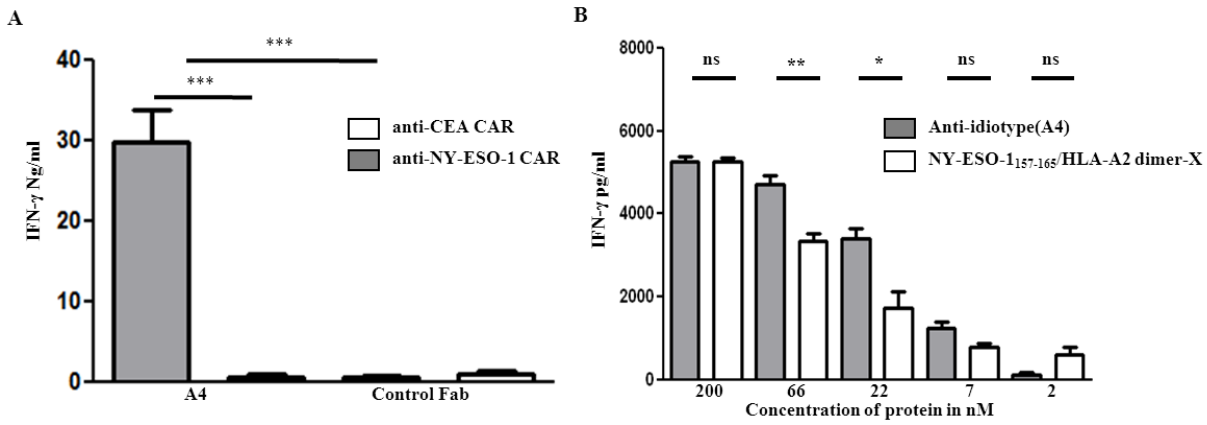
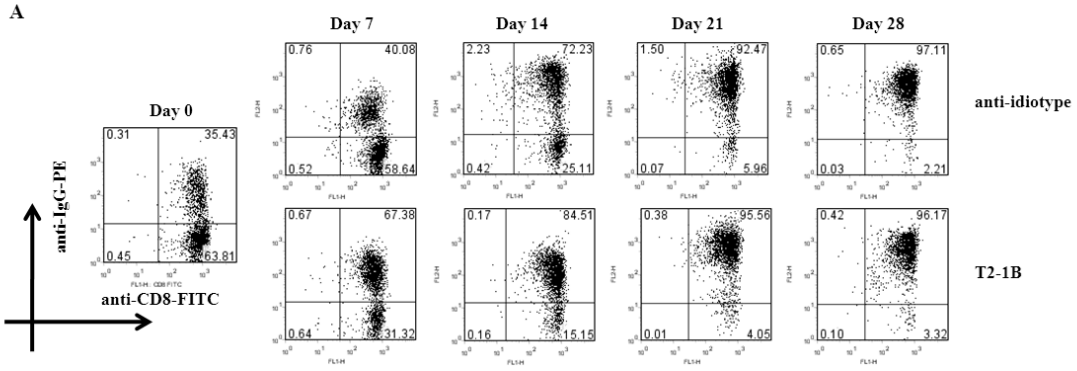


Figure 4-11: Anti-idiotypic Fab dependent activation of anti-NY-ESO-1CAR re-directed CD8+T cells. **A** Human CD8+T cells were retrovirally transduced with anti-NY-ESO-1CAR and anti-CEA CAR, equal number of CAR positive re-directed CD8+ T cells were cultured with plate-coated anti-idiotypic and control Fabs for 24h. As activation marker IFNγ was measured by ELISA in culture supernatant. **B** Equal numbers of recombinant HLA-A2 dimer molecule (pulsed with NY-ESO-$1_{157\text{-}165}$ peptide) anti-idiotypic Fab molecules at different concentrations were coted on culture plates. Anti-NY-ESO-1 CAR re-directed T cells were added to the plate, after 24 hours culture supernatant was collected and secreted IFNγ was measured by ELISA.***P<0.0003

4.5.5 Anti-idiotypic Fab dependent expansion of anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells

Since we demonstrated dose-dependent and antigen-specific activation of re-directed T cells by anti-idiotypic Fab as observed for HLA-A*0201/NY-ESO-$1_{157–165}$ positive cells, we aimed to test anti-idiotypic Fab and target cells for the antigen-specific expansion of re-directed T cells. Receptor-triggered proliferation of re-directed CD8+ T cells was monitored induced by of anti-idiotypic Fab A4 molecule or irradiated T2-1B (50Gy). We compared the antigen specific expansion of anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells with irradiated T2-1B (50Gy) cells or with anti-idiotypic Fab molecules. We monitored changes in CAR positive T cells and cell number over a period of 28 days. Increase in receptor positive cells was identified by surface staining with anti-human IgG antibody and the number was determined by counting viable cells on a weekly basis. Immobilized anti-idiotypic Fab A4 and T2-1B cells specifically increased the proliferation of anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells in contrast to control Fab (figure 12A and B). There was a significantly more rapid expansion of anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells in response to anti-idiotypic Fab A4 Fab as compared to irradiated T2-1B cell stimulation. Considering the number of antigen specific T cells required for the adoptive T cell therapy, anti-idiotypic dependent expansion increases 150 fold of T cell number with in 28days compared with initial cell number and this cell number was double than cells derived by T2-1B cell dependent expansion (Figure 12B).



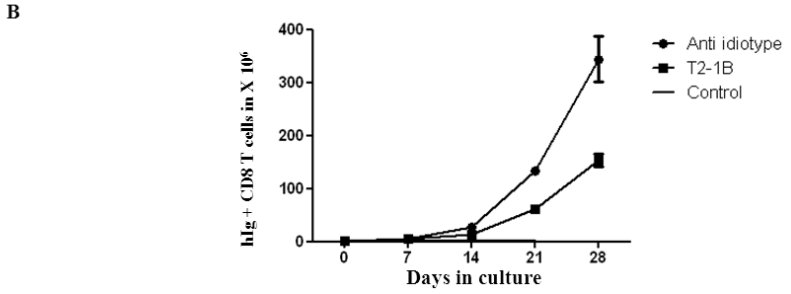


Figure 4-12: Antigen dependent expansion of anti-NY-ESO-1CAR re-directed $CD8^+$ T cells. **A** At day 0, equal numbers of re-directed anti-NY-ESO-1 CD8+ cells were co-cultured with HLA-A*0201/NY-ESO-$1_{157–165}$ peptide expressing T2 cells (T2-1B) in a ratio of 4:1 or with anti-idiotypic Fab A4 (2 µg/ml) coated flasks in presence of rhIL-2 (50 IU/ml). Increase in receptor positivity and cell number was monitored for a period of 4 weeks. Increase in receptor positive T cells was analyzed by flow cytometry using PE-conjugated anti-human IgG1 and FITC-conjugated anti-CD8 mAb. **B** Increase in number of receptor positive cells was counted on weekly basis.

4.5.6 Functional analysis of CAR re-directed CD8+ T cells during *in vitro* expansion

The functional capacity of the *in vitro* expanded anti-NY-ESO-1 CAR (T1) re-directed $CD8^+$T cells in response to antigen-specific stimulation was analyzed. To address this question, we collected anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells at different time points of the expansion phase and co-incubated them with T2-1B (antigen specific) and T2-1C (control) for 4h. Expanded anti-NY-ESO-1 CAR (T1) re-directed T cells were fully functional since they secreted cytokines (IFNγ, TNFα and IL-2) in an antigen-specific manner at day 28 (figure 13). Over time similar antigen-specific cytokine secretion trends were observed in both anti-idiotypic and T2-1B expanded re-directed T cells in response to antigen (figure 14 A and B).

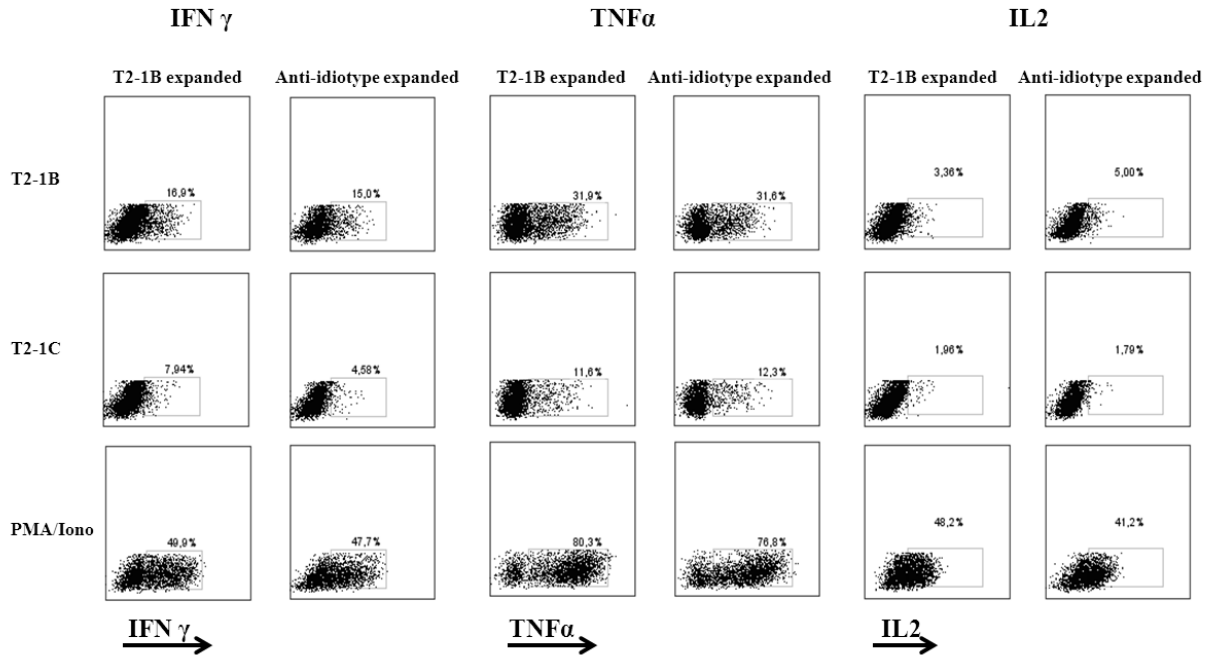


Figure 4-13.1: Comparison of the percentage of poly-functional antigen specific CAR re-directed CD8+ T cells after 28 day of expansion with T2-1B cells or anti-idiotypic Fab A4. Antigen-specific cytokine release was determined by intracellular staining. T2-1B and anti-idiotype Fab A4 expanded anti-NY-ESO-1 CAR

re-directed CD8+ T cells were incubated with T2-1B (antigen specific) and T2-1C (control) cells for 4 hours. As positive control cells were stimulated withPMA/ionomycine. Percentage of IFNγ, TNFα and IL-2 positive cells from T2-1B cell and anti-idiotypic Fab A4 expanded CAR positive CD8+ T cells.

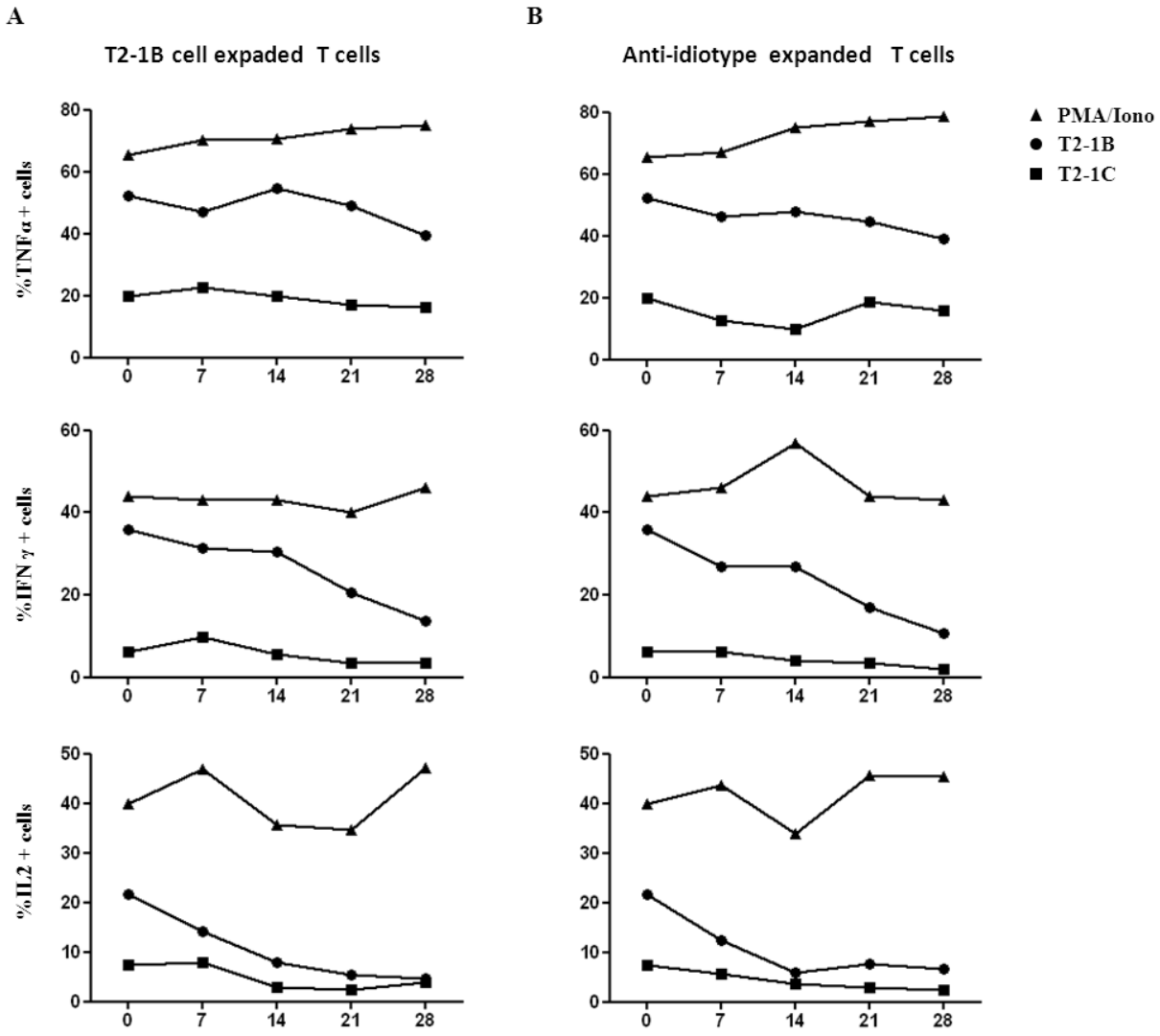


Figure 4-13.2: Activation of expanded anti-NY-ESO-1 CAR re-directed CD8+T cells. Percentage of poly-functional antigen specific CAR re-directed CD8+ T cells were determined by intracellular cytokine staining for a period of 28 days. T2-1B and anti-idiotypic Fab A4 expanded anti-NY-ESO-1 CAR re-directed CD8+ T cells were incubated with T2-1B (antigen specific) and T2-1C (control) cells for 4 hours. As positive control cells were stimulated with PMA/ionomycine. Percentage of IFNγ, TNFα and IL-2 positive cells from T2-1B cell (**A**) and anti-idiotypic Fab A4 (**B**) expanded CAR positive CD8+ T cells at different time points of T-cell culture. (n=2, one representative experiment shown)

4.5.7 Expanded anti-NY-ESO-1 CAR (T1) expressing redirected CD8+ T cells lysed the antigen expressing target cells

We further analyzed cytolytic potential of expanded anti-NY-ESO-1 CAR (T1) re-directed CD8 positive T cells. Co-incubation of expanded anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells with T2-1B and T2-1C cells for 24 hours resulted in antigen-specifically lyses of T2-1B target cells regardless of the expansion protocol (figure 15 A and B), whereas no specific lysis was observed with T2-1C cells.

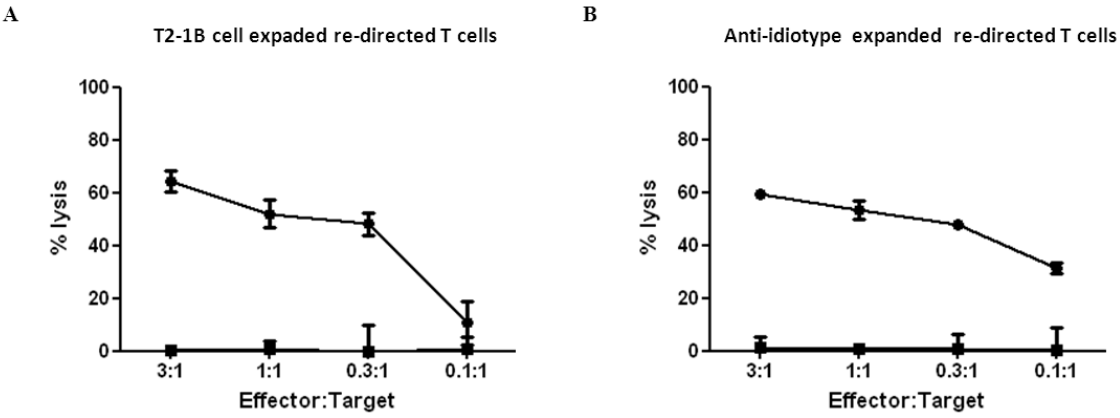


Figure 4-14: Cytotoxicity of expanded re-directed T cells was assessed by measuring viability of target cells by a tetrazolium salt-based XTT assay after 24h. T2-1B (**A**) and anti-idiotypic Fab A4 expanded anti-NY-ESO-1CAR re-directed CD8+T cells (**B**) were incubated with T2-1B (antigen specific, circles) and T2-1C (control, squares) at different effector to target cell ratios.

4.5.8 Phenotypic characterization of expanded re-directed CD8+ T cells

For the phenotypic analysis of expanded anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells, we used a flow cytometric panel to distinguish different populations of central memory (CCR7+, CD62L+), effector memory (CCR7-, CD62L-) (Unsoeld and Pircher 2005) cells to compare the effect of anti-idiotypic Fab and T2-1B cells on phenotypinc change of anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells. The analysis was done with after day 28 *in vitro* expanded re-directed T cells. We have observed highly variable phenotypic difference between experiments. Anti-idiotypic T cell expansion resulted either in high numbers or low numbers of central-memory re-directed T cells depending on the donor.

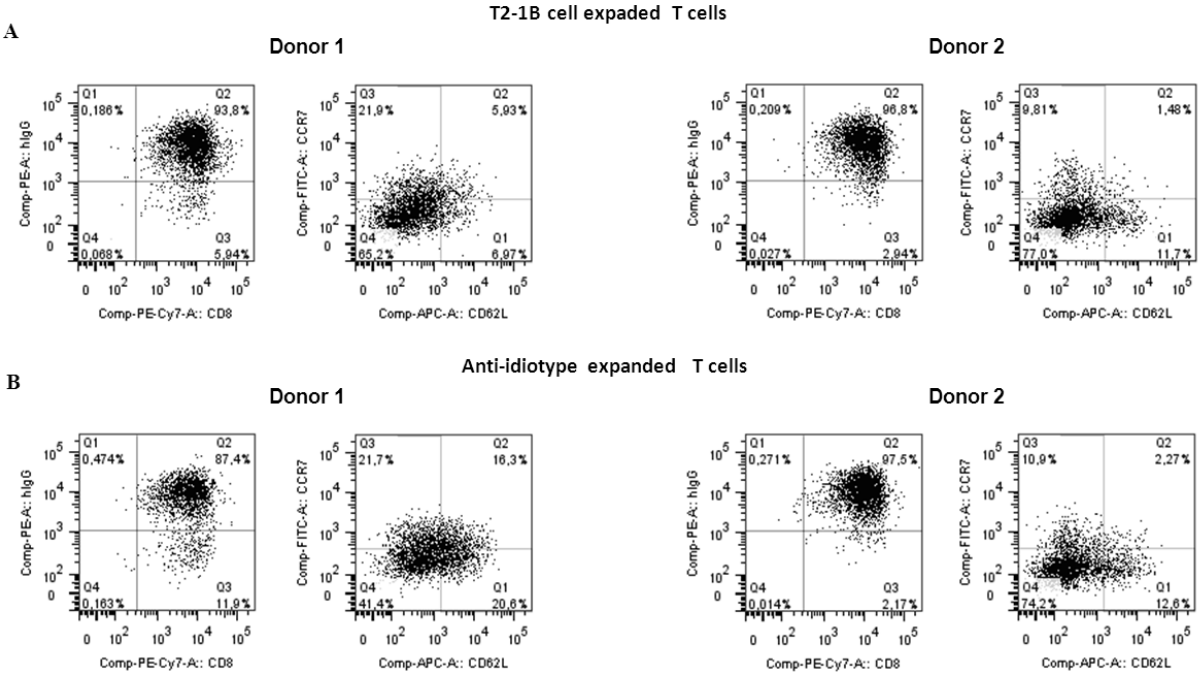


Figure 4-15: CD62L and CCR7 expression on anti-idiotypic and T2-1B expanded anti-NY-ESO-1 CAR (T1) redirected CD8+ T cells. **A** CD62L and CCR7 staining onT2-1B expanded anti-NY-ESO-1 CAR (T1) positive re-directed CD8+ T cells. **B** CD62L and CCR7 staining on anti-idiotypic expanded anti-NY-ESO-1 (T1) CAR positive re-directed CD8+ T cells

4.5.9 Antitumor effect of expanded anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells *in vivo*

To finally prove functionality of antigen-specially expanded re-directed T cells, re-directed CD8+ T cells were assessed in a Winn assay (Dent, Spencer et al. 1989). Winn assays can be used to demonstrate protection in xenograft models. It has been previously shown that freshly transduced anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells exhibit the anti-tumor effect in a protective mouse model (Schuberth, Jakka et al. 2012). In the herein performed Winn assay, NSG mice were subcutaneously injected with U266 cells ($10x10^6$). Co-injection was performed with either $10x10^6$ anti-idiotypic or cell-based expanded anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells. Tumor growth was measured by the volume of the subcutaneous tumors and by measuring human IgE in the serum of mice. The Multiple myeloma U266 cells secret human IgE, which could be used as a surrogate parameter for cell growth. The control group injected with U266 cells alone started secreting human IgE after one week of injection whereas no IgE secretion was observed in anti-idiotypic expanded and T2-1B expanded CD8+ T cell groups. Increase in IgE levels were observed in control group mice up to day 35, whereas no IgE secretion was observed in anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells injected mice. The

growth of the subcutaneous tumor clearly indicated the anti-tumor activity of the expanded anti-NY-ESO-1 CAR redirected CD8+ T cells. However, we have not observed differences between anti-idiotypic expanded and T2-1B expanded anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells regarding anti-tumor activity. These results demonstrated that growth of the Multiple myeloma cell line U266 was suppressed by expanded anti-NY-ESO-1 CAR (T1) re-directed CD8+ T cells in a Winn assay.

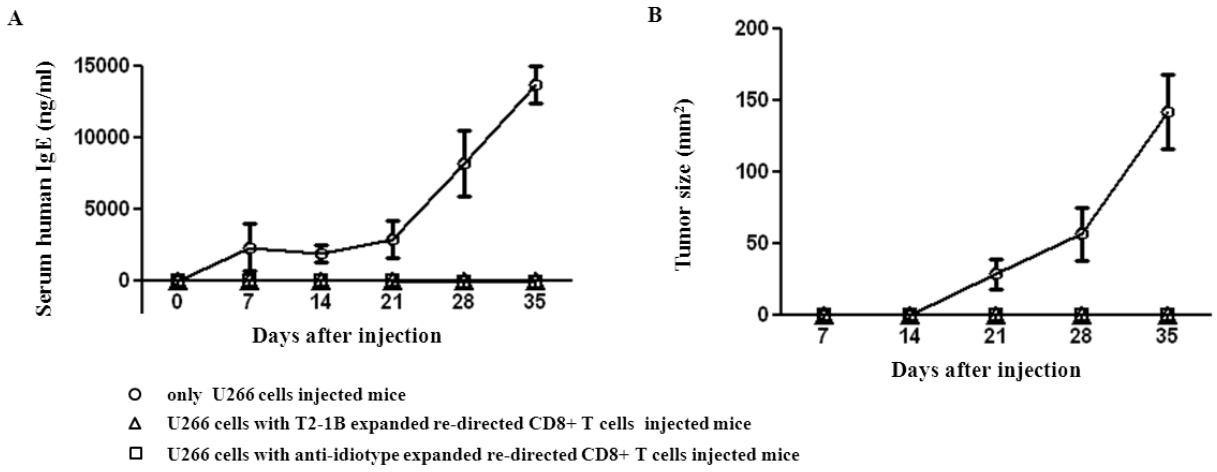


Figure 4-16: Expanded anti-NY-ESO-1 CAR (T1) redirected CD8+ T cells mediate anti-tumor activity in Winn assay. Mice were subcutaneously injected 10 X 10^6 U266 cells with 10 X 10^6 of anti-idiotype, T2-1B expanded anti-NY-ESO-1 CAR redirected CD8+ T cells and PBS control. Tumor growth was monitored by measuring serum IgE levels (A) and tumor size (B) (n=2, one representative experiment shown).

5 Discussion

Cancer immunotherapy aims to eliminate malignant cells by activating the immune system in an antigen specific manner; one way of eliminating malignant cells is by adoptive transfer of tumor specific T cells. Adoptive T cell transfer allows for the characterization and modification of T cells to target tumor cells and expand them *in vitro* in the absence of tumor suppressive mechanisms. Recent clinical trials with re-directed T cells showed promising results in patients with chronic lymphoid leukemia (Porter, Levine et al. 2011). Our study characterized HLA-A2/NY-ESO-$1_{157-165}$ re-directed CD8+ T cells *in vitro* and demonstrated the relevance of receptor affinities for adoptive T cell therapy. We have developed a method for the antigen specific expansion of clinical grade re-directed anti-NY-ESO-1 CAR positive T cells by using anti-idiotypic Fab molecules. Our results indicate *in vitro* expanded anti-NY-ESO-1 CAR (chimeric antigen receptor) re-directed T cells are capable of antigen specific lysis of NY-ESO-1 157-165 transfected T2 cells and inhibition of multiple myeloma tumor growth in xenograft model when analysed by a Winn assay system.

First generation CARs had their antigen-binding domain connected to a transmembrane domain and CD3ζ intracellular signaling domain which showed anti-tumor efficacy *in vitro* by lysing tumor cells but *in vivo* efficacy was limited (Carpenito, Milone et al. 2009). Signaling through CD3ζ alone is not sufficient for the complete activation of re-directed T cells, resulting in a compromised cell proliferation, survival and antigen dependent IL-2 production *in vivo*. In physiological conditions, IL2 production in T cells is initiated by engagement of CD28 on activated antigen-specific T cells to its CD80/86 ligands on APCs (Fraser, Newton et al. 1992). Most of the malignant cells do not express CD28 ligands on their surface which leads to the loss of effectiveness of first generation CAR re-directed T cells *in vivo*. Intracellular signaling domain of CD28 is placed proximal to the CD3ζ in first generation CAR resulted in production of IL-2 and provides antigen-specific signals 1 and 2 in CAR re-directed T cells called second generation CAR (Figure 2-6) (Savoldo, Ramos et al. 2011). HLA-A2/NY-ESO-$1_{157-165}$ peptide complex recognizing anti-NY-ESO-1 scFv (wild type) and anti-NY-ESO-1 T1 scFv (affinity matured) were used to generate second generation CAR re-directed CD8+ T cells. In the present study, we show the expression of anti-NY-ESO-1 CAR constructs on the CD8+ T cell surface (Retroviral mediated gene transfer) and recognize NY-ESO-1157-165 peptide in context with HLA-A2 (figure 5-2).

An effective antigen specific immune response generates different subsets of T cells including central memory, effector memory and effector cells (Sallusto, Geginat et al. 2004). We have reported that CAR re-directed CD8+ T cells consists of different T cell subpopulations

(Schuberth, Jakka et al. 2012). CAR re-directed CD8+ T cells show antigen specific activation and produce pro-inflammatory cytokines IFNγ, TNFα and IL-2 (Figure 5-4). It has been demonstrated that IFNγ, TNFα and IL-2 simultaneously producing T cells provide optimal effector function (Darrah, Patel et al. 2007). Our results from the cytokine analysis of either simultaneous (IFNγ, TNFα and IL-2) or individual cytokine secretion (IFNγ orTNFα or IL-2) by CAR re-directed CD8+ T cells indicate the existence of different phenotypic populations of CD8+ T cell subsets (Schuberth, Jakka et al. 2012).

Tumor antigenic (mostly self-antigens) peptide presented MHC molecules interaction with specific TCRs tend to be of weaker affinity than TCR specific peptides originated from pathogens (Cole, Pumphrey et al. 2007). Selective replacement of one/two amino acids in the antigen binding region of TCRs resulted in increased affinity with antigen specificity (Li, Moysey et al. 2005). Previously, we found that affinity maturation by selective replacement of two amino acids in the light chain of our anti-HLA-A2/NY-ESO-1 157-165 TCR like Fab antibody molecule enhanced the affinity 20 fold (Stewart-Jones, Wadle et al. 2009). We have shown in the present study that both, wild type (anti-NY-ESO-1 CAR) and affinity matured (anti-NY-ESO-1 T1 CAR) chimeric antigen re-directed CD8+ T cells kept their specificity for the HLA-A2/NY-ESO-$1_{157-165}$ peptide. Moreover, higher affinity CAR re-directed CD8+ T cells significantly enhanced the antigen specific cytotoxic T cell activity. These results indicate that antigen specific affinity maturation of TCR like antibodies not only enhances the binding but also functionality of the scFv re-directed CD8+ T cells at lower effector (T cells) and higher target cell (T2-1B) ratios (figure 5-5). We also observed an enhanced antigen specific secretion of IFNγ and granzyme B molecules in *in vitro* culture supernatants with affinity matured scFv re-directed CD8+ T cells which was not observed with any other pMHC molecules.

CD4 and CD8 expression on T helper cells and cytotoxic T lymphocytes (CTLs) is known to play a important role for the sensitivity and activity of T cells to their respective peptide MHC complex (Holler and Kranz 2003; Li, Dinner et al. 2004). Several studies have shown that antigen recognition by chimeric antigen receptors derived from monoclonal antibody is independent of MHC restriction. Furthermore, MHC class I restricted CD8+ and MHC class II restricted CD4+ T cells can be re-directed by chimeric antigen receptors (Hombach, Heuser et al. 2001). In this study, we demonstrate MHC restricted and co-receptor independent activation of chimeric antigen receptor recognizing HLA-A2/NY-ESO-$1_{157-165}$ peptide re-directed T cells. Antigen specific activity of CAR re-directed CD4+ T cells is highly efficient and comparable with those of re-directed CD8+ T cells (Figure 5-6). Notably, increasing peptide density on target cell surface enhanced the T cell activation in both CD4+ and CD8+ re-directed T cells. It has been shown that CD4 and CD8 co-receptors play an important role in stabilizing the interaction between

TCR and peptide MHC complex under physiological conditions (Artyomov, Lis et al. 2010). Contrary to the findings, we observed that chimeric antigen receptors, derived from high affinity TCR like antibody targeting HLA-A2/NY-ESO-$1_{157-165}$ peptide, re-direct the T cells in a co-receptor independent manner. These findings are similar to that of high affinity MHC class I restricted TCRs targeting tumor antigens which also function in co-receptor independent manner (Schmid, Irving et al. 2010).

It has been shown that the HLA-A2/NY-ESO-$1_{157-165}$ peptide complex expression is enhanced after treatment with 5-aza-2-deoxycytidine (DAC) in different types of cancer cells (Guo, Hong et al. 2006). Several studies used DAC treatment to trigger the expression of p157-165 MHC complexes and, additionally, enhanceed the NY-ESO-1 specific CTL recognition (Coral, Sigalotti et al. 2002). The efficient anti-NY-ESO-1 T1 CAR re-directed CD8+ T cells recognition of DAC treated breast cancer cell line (MCF7) demonstrates that *de novo* synthesized NY-ESO-1 is functional and it allows for specific T cell recognition of MCF7 cells that are otherwise completely resistant to anti-NY-ESO-1 T1 CAR re-directed CD8+ T cell mediated lysis. Moreover, activity of anti-NY-ESO-1 T1 CAR was enhanced in the Multiple myeloma cell line (U266) upon DAC treatment (figure 5-7). DAC treatment induced or enhanced the expression of NY-ESO-1 protein rather than increased expression of HLA class I molecules on the cell surface of tumor cells (Coral, Sigalotti et al. 1999). Thus, the anti-NY-ESO-1 T1 CAR re-directed CD8+T cell mediated lysis induced or enhanced by DAC treatment represents a direct consequence of NY-ESO-1 expression leading to sufficient amounts of immunogenic peptide loaded onto pre-existing HLA-A2 molecules in MCF7 cells which enabled efficient recognition of target cells by antigen specific T cells (Christinck, Luscher et al. 1991).

Although cancer immunotherapy with tumor infiltrating lymphocytes (TILs) or re-directed T cells has shown significant activity in clinical settings, the expansion procedure requires simplification to allow for a broad range of applications (Ye, Loisiou et al. 2011) and, finally, to improve clinical outcome. It has been shown that sufficient numbers of T cells can be generated for the adoptive T cell therapy either by expanding TILs or re-directed T cells *in vitro* (Porter, Levine et al. 2006). In our study, we investigated the possibility of using anti-idiotypic Fab antibody molecules specific for the anti-NY-ESO-1 T1 CAR to boost the selective expansion of CAR re-directed CD8+ T cells. Anti-Idiotypic Fab antibody mediated expansion could be a valuable method to obtain sufficient numbers of tumor specific T cells for tumor targeting in the concept of adoptive T cell transfer. Our results were comparable with tumor specific T cells generated by other *in vitro* expansion protocols (Brimnes, Gang et al. 2012). In order to obtain sufficient numbers of tumor specific T cells for adoptive T cell therapy, tumor reactive $CD8^+$ CTLs were either expanded with anti-human CD3/CD28 coated beads or expanded with APCs cells with weekly

stimulation (Li and Kurlander 2010). Expansion of T cells with anti-idiotypic Fab antibody molecules has several advantages compared to other expansion procedures. The most appealing advantage is these of purification and production of GMP grade anti-idiotypic Fab antibody molecules for the *in vitro* stimulation and expansion. Furthermore, anti-idiotypic Fab antibody molecules specifically activate CAR grafted CD8+T-cells from heterogeneous populations unlike polyclonal stimulation with anti CD3&CD28 antibodies (Teschner, Wenzel et al. 2011). Even though antigen specific T cells can be generated by loading peptides on artificial antigen presenting cells (aAPCs) or APCs, anti-idiotypic Fab antibody molecules provide cell-free antigen specific expansion (Yuan, Gallardo et al. 2006). In this present study we observed that both anti-idiotypic Fab antibody molecules and antigen transfected T2 cells (T2-1B) can be used for the specific expansion of CAR grafted $CD8^+$ T-cells. We also have demonstrated that anti-idiotypic Fab antibody molecules depended expansion is significantly more efficient than antigen transfected cell based expansion. Another advantage of using anti-idiotypic Fab antibody molecules over APCs is that the generation of antigen specific APCs requires a specialized laboratory and appropriate quality control (Papanicolaou, Latouche et al. 2003). We have demonstrated that anti-idiotypic Fab molecules bind to immobilized 3M4E5 IgG, to cell surface expressed scFv CARs on transfected 293T cells and freshly transduced human CD8+ T cells (figure 5-8), respectively. These anti-idiotypic Fab antibody molecules were comparable with HLA-A*0201/NY-ESO-$1_{157–165}$ tetramer for the detection of surface expression of anti-NY-ESO-1 CAR T1 and compete with the HLA-A2/NY-ESO-$1_{157-165}$ tetramer for the CAR, which is expressed on transduced CD8 +T cells (figure 5-9&10).

In addition to specific binding and competition with the antigen, we also compared the immobilized anti-idiotypic Fab antibody molecule with immobilized HLA-A*0201/NY-ESO-$1_{157–165}$ complex. Anti-idiotypic Fab antibody molecules can efficiently activate anti-NY-ESO-1 T1 CAR re-directed CD8+T-cells. Furthermore, we observed significantly higher activation potential of anti-idiotypic Fab antibody molecules with equal numbers of HLA-A*0201/NY-ESO-$1_{157–165}$ complexes (figure 5-11). Several studies have used CD3/CD28 beads or autologous APCs to successfully generate tumor specific T cells for adoptive T cell therapy (Rasmussen, Borelli et al. 2010). Peptide stimulation prior to the CD3/CD28 bead stimulation further increases the number of antigen specific T cells. We also observed similar results that anti-idiotypic Fab antibody dependent expansion specifically enhanced the number of anti-NY-ESO-1 T1 CAR re-directed CD8+ T cells, more than that by antigen transfected T2-cells (figure 5-12).

Adoptive transfer of tumor-specific memory cells T cells has been shown to be superior in tumor protection (Kim, Teh et al. 2009). Antigen specific T cells expanded *in vitro* with aAPCs comprise of central memory (CCR7+ CD62L+) and effector memory populations (CCR7-

CD62L-), adoptive transfer of these *in vitro* expanded T cells showed establishment of anti-tumoral immunological memory (Chapuis, Thompson et al. 2012). We observed similar results with anti-idiotypic Fab antibody dependent expansion of anti-NY-ESO-1 T1 CAR re-directed CD8+ T cells since the expanded population consisted of a central memory population (CCR7+ CD62L+) and effector memory population (CCR7- CD62L-). Moreover, we observed an increase of the central memory population with anti-idiotypic Fab antibody dependent expansion (figure 5-15). Repeated activation of T cells would result in activation induced cell death as observed in chronic viral infection and several tumor models (Saff, Spanjaard et al. 2004). We have observed a decrease in the antigen specific cytokine secretion over time, but antigen specific lysis of antigen expressing target cells was maintained (figure 5-13). T-cells expanded by anti-idiotypic Fab antibody show enhanced functional competence over other protocols when cytotoxicity and cytokine secretion are compared. Even though cells demonstrate cytotoxic potential after 28 days of expansion (figure 5-14), unfortunately, cytokine secretion decreases after 14 days of expansion. T-cells (14 days expanded) produce IFNγ, TNFα and IL-2 cytokines upon antigen specific stimulation which indicates the existence of memory T-cell population. We observed similar effect with anti-idiotypic and T2-1B cell dependent expansion. Moreover, anti-idiotypic Fab antibody dependent expansion results in a 2 fold increase of numbers of 3M4E5 CAR grafted T-cells than with T2-1B cell dependent expansion at day 14, which is 25 fold higher than that of starting population.

Multiple myeloma (MM) is incurable neoplastic transformed plasma cells (Kyle and Rajkumar 2008). It has been shown that conventional therapies may target plasma cells to reduce the tumor burden but they were ineffective in eradicating the disease (Matsui, Wang et al. 2008). Our previous study showed anti-tumor effect of anti-NY-ESO-1 T1 CAR in *in vivo* NSG mice model (Schuberth, Jakka et al. 2012). Functionality of the anti-idiotypic Fab antibody expanded T cells *in vivo* was demonstrated by co-injecting subcutaneously 10 X 10^6 U266 cells along with equal number of expanded re-directed T cells. It has been shown that growth of U266 cells in NSG mice can be monitored by measuring serum hIgE as a surrogate marker (Miyakawa, Ohnishi et al. 2004), and tumor volume was measured with caliper. Serum IgE levels were detected in mice with U266 PBS control after 7 days of injection whereas hIgE was not detected in mice injected with expanded CD8+ T cells even after 35 days of injection. Furthermore, we observed the tumor growth in U266 PBS control mice and they correlated with the increase of serum hIgE levels (5-16). Our results were comparable with other T cell expansion protocols.

Finally, our data demonstrate that anti-NY-ESO-1 CAR re-directed T cells have higher affinity, exhibit strong anti-tumor activity and the activity is enhanced by the increase in the endogenous NY-ESO-1 peptide by de-methylating agent. Re-directed CD8+ T cells are composed of

different T cell populations like central memory and effector memory cells and showed antigen specific functionality *in vitro*. Required numbers of tumor specific CAR grafted $CD8^+$T-cells can be generated by using anti-idiotypic Fab antibody molecules for adoptive T cell transfer and yields higher number of tumor specific T cells compared to T cells derived by T2-1B cell dependent expansion. After 28 days of expansion, we show the functionality of these expanded T-cells is maintained as good as tumor cell expanded T cells.

Taken together these findings indicate usage of anti-idotypic Fab antibody dependent cell free system for adoptive T-cell transfer studies as a novel method for the expansion of clinical grade TCR like antibody CAR grafted $CD8^+$T-cells.

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